Immunological Characterization of the HIV-Tuberculosis associated Immune Reconstitution Inflammatory Syndrome

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DECLARATION

I, Rabecca Tadokera do hereby declare that the work on which this thesis, entitled:

Immunological characterization of the HIV-Tuberculosis associated Immune Reconstitution Inflammatory Syndrome

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"Scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like radium, a benefit."- (Marie Curie, 1867-1934)

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ABSTRACT

While the integration of anti-TB and cART therapies is associated with substantial clinical improvement in the majority of patients, HIV-Tuberculosis associated Immune Reconstitution Inflammatory Syndrome (TB-IRIS) has been shown to occur in a significant subset of these patients. TB-IRIS is an inflammatory complication of the combined treatments for HIV-1 and tuberculosis, which is being reported increasingly, particularly in areas endemic to both diseases. This work aimed to characterise the immunopathogenesis of paradoxical HIV-Tuberculosis associated immune reconstitution inflammatory syndrome.

Patients were recruited at health centres in Cape Town. TB-IRIS patients were diagnosed according to a validated consensus case definition while controls were similar HIV-TB patients who were prescribed the same duration of cART but who did not develop TB-IRIS. PBMC were cultured in the presence or absence of heat killed *M. tuberculosis* (MTB) H37Rv for 6 and 24 hours. RNA from cultures was analysed by quantitative RT-PCR and culture supernatant and the corresponding serum analysed by multiplex for various immune mediators. Stimulation with *M. tuberculosis* increased the transcript abundance for many of the pro- and anti-inflammatory cytokines analysed. IL-1 Beta, IL-5, IL-6, IL-10, IL-13, IL-17A, IFN-gamma, GM-CSF and TNF transcripts were significantly higher in stimulated TB-IRIS cultures at either the 6 or 24 hour time points ($p_{corr.} \leq 0.05$). Gene induction for IL-6, IL-10, IL-12p40, IL-13, IL-17A and IFN-gamma was significantly greater in TB-IRIS patients ($p_{corr.} \leq 0.05$). Luminex analysis in the corresponding tissue culture supernatants showed the largest and significant fold differences in IL-1Beta, IL-2, IL-6, IL-8, IL-10, IL-12p40, IFN gamma, GM-CSF and TNF ($p_{corr.} \leq 0.04$). Serum analysis showed that the levels of TNF, IL-6 and IFN-gamma were significantly higher in TB-IRIS patients ($p_{corr.} \leq 0.02$). Furthermore, the serum levels of IL-6 and TNF decreased significantly in prednisone treated ($p_{corr.} \leq 0.04$)

but not in placebo treated TB-IRIS patients. Many pro- and anti-inflammatory cytokine transcript and protein levels are elevated in TB-IRIS patients indicating that cytokine release contributes to the immunopathology. IL-6 and TNF were elevated under all conditions and decreased in serum during corticosteroid therapy. These results suggest that specific blockade of IL-6 and TNF may be a novel and rational approach to immunomodulation in TB-IRIS.

The role of tissue degrading enzymes and the effect of corticosteroid treatment was investigated by analysing MMP gene expression and protein concentration *in vitro* and *in vivo* in TB-IRIS patients and relevant controls. Increased expression of MMP-1, -3, -7, -10 and MMP-12 genes was observed in TB-IRIS ($p \leq 0.05$) compared to controls. Protein secretion in 24 hour stimulated cultures was also higher in TB-IRIS for MMP-1, -3, -7, and -10 ($p \leq 0.008$). Circulating MMP-7 concentrations were significantly elevated in the serum of TB-IRIS. Furthermore, a 4 week course of corticosteroid therapy suppressed MMP-7 gene expression and serum levels in TB-IRIS. This study showed the association of TB-IRIS with a distinct pattern of MMP activation with MMP-7 being the most consistently elevated and was modulated by prednisone therapy. Modulation of dysregulated MMP activity, particularly MMP-7 may represent a promising and novel therapeutic approach to alleviate TB-IRIS.

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AIDS	Acquired Immuno-Deficiency Syndrome
ANOVA	Analysis of Variance
APC	Antigen presenting cell
ARDS	Acute Respiratory Distress Syndrome
ART	Antiretroviral Therapy, highly Active Antiretroviral Therapy
BAL	Brochoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
cART	Combination Antiretroviral Therapy
CD4	Cluster of differentiation 4 (T helper cells)
CD8	Cluster of differentiation 8 (Cytotoxic T cells)
CD28	CD28 receptor
CD45RO+	CD45 receptors
CD4+CD45 RO+	memory CD4+ T cells
CD45RA+CD62L+	naive CD4+ T cells
CCL-	beta chemokine, chemokine motif ligand
CXCL-	alpha chemokine
CI	Confidence interval
COPD	Chronic Obstructive Pulmonary Disease
CO_2	carbon dioxide
CRP	Creatine reactive protein
CT	Cycle Threshold
Δ Ct	delta Ct, Difference in Cycle Threshold
°C	degree Celsius
DNA	Deoxyribonucleic acid

DTH	Delayed Type Hypesensitivity
ECM	Extracellular Matrix
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunosorbent spot assay
ESAT-6	6kDa Early secretory antigenic target
F	female
FoxP3	Fox head box 3, marker of regulatory cells
g	centifugal force, gram
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H_2SO4	Sulphuric acid
H37Rv	TB antigen
IFN-γ	Interferon gamma
HIV	Human Immuno-Virus
HIV-1	Human Immuno-virus subtype 1
HRP	Horse radish peroxide
IQR	Interquatile range
INSHI	International Network of HIV-associated IRIS
IFN-γ	Interferon gamma
IRIS	Immune reconstitution Inflammatory Syndrome
IP-10	Interferon gamma inducible protein
IL-	Interleukin
JC virus	poliomavirus
kg	kilogramme
KIR	Killer Immunoglobulin Receptor
M	Male

MAC	*Mycobacterium Avium Complex*
mAb	monoclonal antibody
MAI	*Mycobacterium Avium Intracellulare*
MCP-1	Macrophage Inflammatory Protein-1
MCP-2	Macrophage Inflammtory Protein-2
MDR-TB	multi-drug resistant TB
MHC	Major Histo compatibility complex
ml	millilitre
mg	milligramme
MMP	matrix metalloproteinase
MIP-	Macrophage Inflammatory protein
MOI	multiplicity of infection
mRNA	messenger RNA
MTB	*Mycobaterium tuberculosis*
Na	Sodium
NA	Not Applicable
ND	not determined, not detected
ng	nano gramme
NK cells	Natural Killer cells
Nm	nanometer
OI	Opportunistic Infection
OD	optical density
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pg	pico gramme

PBS	Phosphate buffered saline
PPD	Purified protein derivative
qPCR	quantitative PCR, Real-Time PCR
RANTES	Regulated upon Activation, normal T cell Expressed and secreted, chemokine
RNA	Ribonucleic acid
rpm	rotations per minute
RT-PCR	Real-time PCR
RT	Room Temperature
S	Stimulated sample
SD	Standard deviation
sIL-R	soluble interleukin receptor
Std	Standard
sIL-R	Soluble interleukin receptor
TB	Tuberculosis
TB-IRIS	Tuberculosis associated IRIS
TGF-β	Transforming growth factor-beta
TGN1412	immunomodulatory drug, CD28-Super mAB
Th	T helper cells
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
Th17	Type 17 helper cells, IL-17 producing T cells
TIMP	Tissue inhibitor of matrix metalloproteinase
TMB	chromogenic substrate
TNF	Tumour necrosis factor
Treg	Regulatory T cells

TST	Tuberculin skin test
U	unstimulated sample
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation

1 CHAPTER 1: INTRODUCTION

1.1 The HIV epidemic

Since the time of its initial description more than two decades ago, the human immunodeficiency virus (HIV-1) continues to be a major health challenge, spreading relentlessly all around the globe (Sharma, Mohan et al. 2005). Over 33 million people are infected with the virus that causes the acquired immunodeficiency syndrome (AIDS) and more than 67% (22.5 million in 2009) of the HIV-infected population worldwide is from the African region (WHO 2010). In 2009, at least 2.6 million people were newly infected with HIV-1 globally, translating to over 7000 new infections a day. While more than 1.8 million AIDS-related deaths were recorded in the same year, about 1.3 million of these were from Sub-Saharan Africa (WHO 2010).

1.2 Epidemiology of Tuberculosis

Tuberculosis (TB) is an ancient bacterial disease, which remains a major health problem in much of the developing world. The disease is caused by infection with *Mycobacterium tuberculosis* (and occasionally by *Mycobacterium bovis* and *Mycobacterium africanum*) (Zumla and Grange 2001) and spreads almost exclusively by the respiratory route. Tuberculosis infection occurs when a person carries tuberculle bacilli which if kept under control by the immune system, are capable of persisting in a dormant or latent state in the tissues for many years without causing disease in more than 90% of infected persons (Harries, Maher et al. 2004). More than a third of the world's population is *Mycobacterium Tuberculosis* infected with 95% of the cases and 98% of all TB deaths occurring in

developing countries. Worldwide, more than 1.7 million people die from tuberculosis every year. In 2009 alone, more than 9.4 million incident cases of TB were recorded globally (equivalent to 137 cases per 100 000 population. As shown in Figure 1, southern Africa has the highest prevalence of HIV in new TB cases, and thus has the worst burden of HIV/TB coinfections as reported in 2009 (WHO 2010).

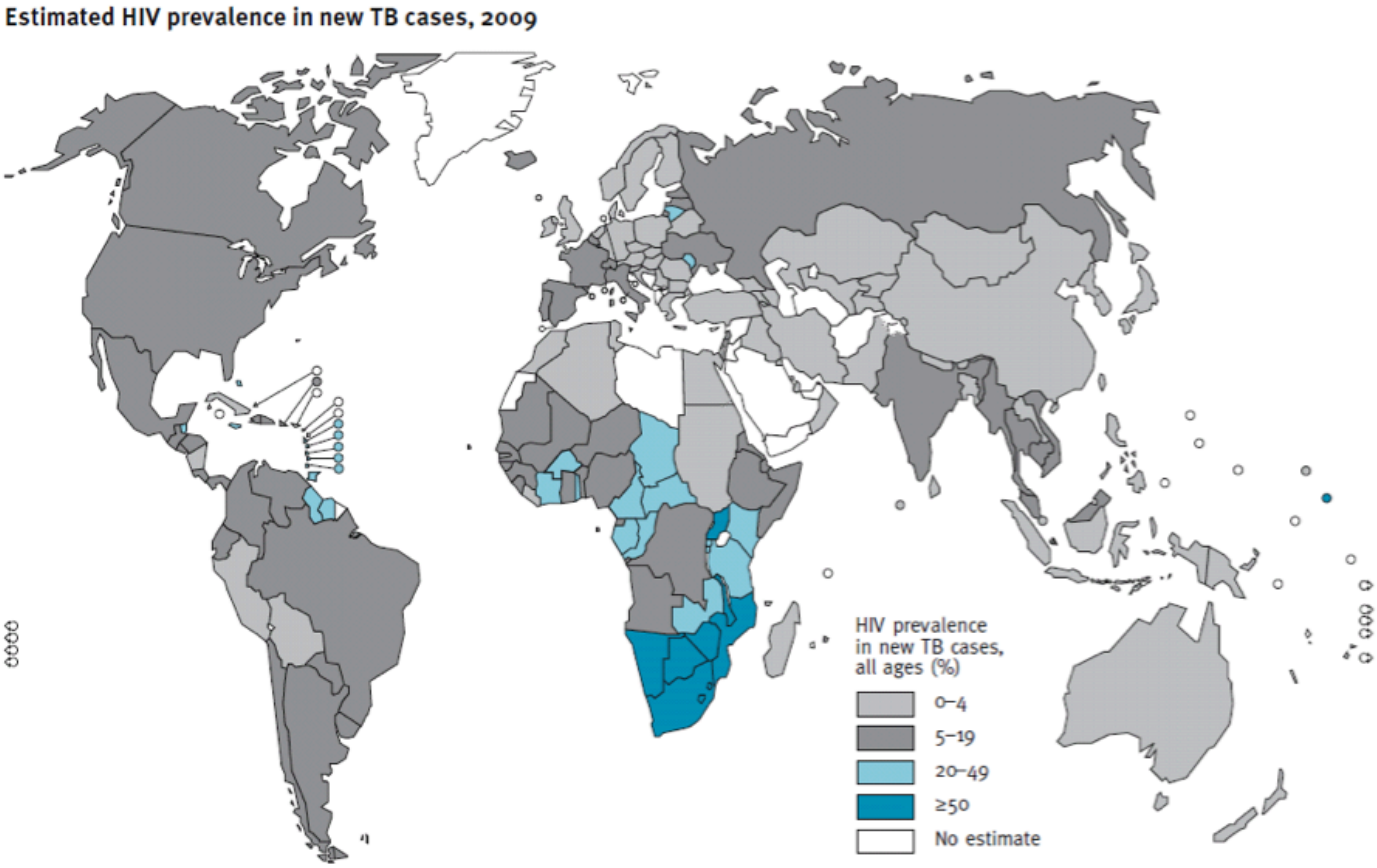


Figure 1 Map showing world distribution of TB/HIV coinfections

(2010 Global TB Control Report)

1.3 The HIV/TB Association

HIV infection is the strongest of all known risk factors for the development of tuberculosis. The global resurgence of TB has largely been driven by the HIV-1 pandemic. The two diseases have a synergistic interaction, each accentuating the progression of the other. Th1 type immune response characterised by adequate cell-mediated immunity is the crucial host defence against *M. tuberculosis* (Schluger and Rom 1998). HIV infection primarily affects those components of the host immune response responsible for cell-mediated immunity, and this results in increased activation of latent *Mycobacterium tuberculosis* (MTB) infection, while HIV-infected persons are more susceptible to new tuberculosis infection (Corbett, Watt et al. 2003).

In persons infected with MTB only, the lifetime risk of developing TB ranges between 5% and 10%, while the annual risk of tuberculosis amongst HIV-1 infected persons in high incidence settings can exceed 20% (Corbett, Watt et al. 2003). Globally, 9% of all new TB cases (31% in Africa) in adults were attributable to HIV/AIDS, as were 12% of the 1.8 million deaths from TB in the year 2000 (Sharma, Mohan et al. 2005). About a third of the HIV-infected population worldwide is co-infected with *Mycobacterium tuberculosis* and this accounts for about 14 million people world- wide. Thus, the dual epidemic of (HIV) and tuberculosis (TB) remains a formidable health challenge, particularly in sub-Saharan Africa (Maartens and Wilkinson 2007). The South East Asia region of the World Health Organisation (WHO) accounts for nearly 40% of all tuberculosis cases globally and 18% of the world's HIV-infected persons also live in this region. Thus, the social and economic implications of the two epidemics on the economies of the developing world cannot be over-emphasised. Table 1 below is a summary of the statistics of TB/HIV co-infections by WHO regions.

Table 1 Numbers of Adults (15-49 yr) co-infected with HIV and TB in WHO regions as of year 2000

Adapted from Harries et al WHO, 2004

WHO Region	Number of people co-infected with HIV and TB (thousands)	% of Global Total
Africa	7979	70
Americas	468	4
Eastern Mediterranean	163	1
Europe	133	1
South-East Asia	2269	20
Western Pacific	427	4
Total	11440	100

1.4 Combination Antiretroviral Therapy (cART)

1.4.1 HIV and cART

Since the advent of highly-active anti-retroviral therapy or Combination Antiretroviral Therapy (cART) in 1996, a combination of at least three drugs that typically includes either a protease inhibitor (PI) or a non-nucleoside-analogue reverse transcriptase inhibitor (NNRTI) and two nucleoside analogue reverse-transcriptase inhibitors (NRTIs), the prognosis of HIV-1-infected patients has substantially improved (Egger, Smith et al. 1997; Hogg, Yip et al. 1999). In general, the introduction of cART has led to a sustained reduction in the incidence of AIDS-related opportunistic infections and a substantial reduction in HIV-associated

mortality and a reduced risk of tuberculosis in HIV-1 infected persons of 59-80% (Egger, Smith et al. 1997; Badri, Wilson et al. 2002; Maartens and Wilkinson 2007). Integrated antitubercular and cART (as opposed to sequential) therapies are associated with a mortality reduction of greater than 50% (Abdool Karim, Naidoo et al. 2010).

In the majority of patients, successful commencing of cART is associated with a drop in plasma viral load to below detectable limits (> 90%) within the first few months. This allows for the reappearance of immune effector cells, typically CD4 T cells, which are usually depleted during HIV infection (Lawn and Wilkinson 2006). The restoration of CD4 cell recovery during cART usually occurs in two principal phases. The initial phase is characterised by a dramatic rise in CD4+ cells within the first 1-2 weeks of commencing cART and is a result of the redistribution of activated CD4+ CD45RO+ memory cells previously sequestered in lymphoid tissue (Autran, Carcelain et al. 1997; Evans, Bonnez et al. 1998; Carcelain, Debré et al. 2001). Following this phase is a more sustained rise in naïve CD4+ T cells, which is thymus-dependent and is associated with the expansion of naïve CD45RA+CD62L+ cells. This second phase is more gradual and persists for 1-2 years in many patients. The increase in circulating CD4 cell numbers is also associated with an improvement in effector function, the extent of which is directly related to the degree of viral load suppression and the CD4 cell counts in the longer term (Lederman 2001). These findings are supported by a more recent study by Wilkinson et al in *M. tuberculosis* sensitized HIV-infected patients documenting the reconstitution of different cell types in cohort of patients who were followed up for 48 weeks. In this study, a decrease in viral load and an increase in CD4 counts, with a significant expansion of central memory cells by 12 weeks, and expansion of naive T cells by 36 weeks was observed (Wilkinson, Seldon et al. 2009). These findings support the hypothesis that the initial CD4+ count recovery is memory cell mediated, while the subsequent increase in CD 4 count is due to naive T cell expansion.

1.4.2 **cART and IRIS**

The major clinical benefits of cART result from a gradual restoration of pathogen-specific immune responses. However, during the initial months of cART immune reconstitution is complicated by adverse clinical phenomena in which either previously subclinical infections are "unmasked" or pre-existing partly treated opportunistic infections clinically deteriorate (Lawn and Wood 2005; Meintjes, Lawn et al. 2008; Meintjes, Rabie et al. 2009). Clinical deterioration has been reported in between 11 and 43% of HIV-infected patients commencing cART. These clinical phenomena are thought to result from immunopathological host inflammatory responses being "switched on" as the immune system recovers (Lawn and Wood 2005). Several terms can be used interchangeably to describe this phenomenon such as immune reconstitution syndrome, immune reconstitution inflammatory syndrome, immune restoration disease, immunorestitution disease, and immune reconstitution phenomena. In this thesis, the term Immune Reconstitution Inflammatory Syndrome (IRIS) will be used when referring to this condition.

While IRIS has become more frequent with the advent of cART in HIV-infected individuals, it is not a new phenomenon, nor is it specific to HIV-infected individuals receiving cART. The potential for IRIS exists whenever patients who have been severely immunocompromised have rapid restoration of immune function (Cheng, Yuen et al. 2000). However, the frequency of IRIS has dramatically increased since the introduction of triple antiretroviral therapy, particularly its introduction to the developing world, where patients commence cART when already highly immunosuppressed, and where there is often a high pathogen burden, such as tuberculosis and *Cryptococcus* in Southern Africa (Keiser, Anastos et al. 2008).

1.5 The Immune Reconstitution Inflammatory Syndrome (IRIS)

1.5.1 The history of IRIS

The first indication that the restoration of pathogen specific immunity could lead to immunopathological reactions was reported in 1992, when atypical presentations of *Mycobacterium avium* complex (MAC) were seen in patients on zidovudine antiretroviral monotherapy (French, Mallal et al. 1992). MAC as an opportunistic infection usually presents as disseminated infection, and is associated with anergy towards mycobacterial antigens. However, in the case of IRIS, MAC infection presented as localized infection, and was associated with the recovery of delayed type hypersensitivity (DTH) reaction to mycobacterial antigens (French, Mallal et al. 1992). IRIS typically occurs within the first few months of initiating cART and can be associated with various bacterial, invasive fungal and chronic viral infections. Among the pathogens commonly associated with IRIS are mycobacterial infections such as *Mycobacterium Avium complex* (MAC), *Mycobacterium tuberculosis* and *Mycobacterium leprae* as well as viral infections such as, herpes viruses, hepatitis B and C virus, and JC virus (Shelburne, Hamill et al. 2002; French, Price et al. 2004; Lawn and Wilkinson 2006). IRIS has also been described in other non-HIV related settings in which rapid restoration of immune function occurs, such as withdrawal of immunosuppressive therapy or after stopping cancer chemotherapy resulting in recovery from neutropenia (Cheng, Yuen et al. 2000).

1.5.2 HIV-Tuberculosis associated-IRIS

Despite the diverse association and presentations of IRIS, HIV-tuberculosis associated IRIS (TB-IRIS) remains most commonly occurring form of immune reconstitution inflammatory syndrome worldwide and is particularly common in countries with a huge burden of both

diseases (Burman, Weis et al. 2007). Manifestations of TB-IRIS range from mild symptoms such as fever, to life-threatening conditions such as respiratory failure. In many case, the clinical manifestations of TB-IRIS resemble the original disease, such as fever, malaise, weight loss, cough etc. Common presenting features include severe fever, cervical and intra-thoracic lymphadenopathy, and pulmonary infiltrates (Dhasmana, Dheda et al. 2008). Tuberculomas, meningitis, cold abscess and ascites are other less common manifestations of the syndrome, which have been reported.

1.5.3 **Defining HIV-TB associated IRIS**

Due to the diverse presentation of the condition, there have been difficulties in clearly defining IRIS. There is no investigation or biological test that confirms the diagnosis of paradoxical tuberculosis IRIS. Thus, confirmation of the disease relies heavily upon case definitions incorporating clinical and laboratory data (Dhasmana, Dheda et al. 2008). In most cases, diagnosis of the condition is mainly based on a diagnosis of exclusion, consistent history and the exclusion of alternative diagnoses such as non-compliance with treatment for the opportunistic infection (OI), the emergence of a new OI, or drug toxicity (Meintjes, Lawn et al. 2008). In areas where drug-resistant tuberculosis is frequent, extra efforts have to be made to exclude drug resistance as a cause for deterioration. To some extent, the lack of consensus case definitions and clear diagnosis has until recently hindered research on IRIS. General case definitions have been previously proposed, but were not validated and so there has been no consensus on their validity and applicability, particularly in resource-constrained settings with limited laboratory facilities.

To this end, the International Network of HIV-associated IRIS (INSHI) has now modified the previously proposed case definitions for IRIS to make them applicable in resource-limited

settings. The INSHI definitions have since been independently validated by various international research groupings (Manosuthi, Tieu et al. 2009; Eshun-Wilson, Havers et al. 2010; Haddow, Moosa et al. 2010). This definition has 3 main components which include the (i) primary or antecedent requirements, (ii) clinical criteria which include major or minor criteria and (iii) exclusion of possible diagnosis as confirmatory criteria in the definition of paradoxical TB-IRIS (Meintjes, Lawn et al. 2008). The primary criteria in the diagnosis of paradoxical TB-IRIS are the microbiological confirmation of tuberculosis or strong clinical evidence of TB before starting cART in addition to evidence of an initial response to anti-tubercular therapy prior to the initiation of cART. Clinical criterion include that the onset of IRIS should be within 3 months of starting cART and one of the major criterion or two of the minor criteria. The major criteria include new or worsening lymph nodes or cold abscesses and radiological features while minor criteria include worsening of clinical symptoms. The exclusion of alternative explanations for clinical deterioration such as treatment failure, drug resistance, poor adherence or drug toxicity or other opportunistic infections form the third component of the INSHI case definition for paradoxical TB-IRIS (Meintjes, Lawn et al. 2008).

The major revisions incorporated in the INSHI case definitions which make them applicable in resource limited settings are the omission of changes in plasma viral load/CD4 cell counts as necessary criteria in confirming IRIS and the inclusion of a time frame for onset of clinical manifestations for a diagnosis of tuberculosis-associated IRIS to be made (Meintjes, Lawn et al. 2008).

1.5.4 **Categorising TB-IRIS**

HIV-Tuberculosis-associated-IRIS (TB-IRIS) is recognised to present temporally as one of two main forms i.e. either as (i) paradoxical TB-IRIS or (ii) unmasking TB-IRIS. Figure 2 below is a schematic diagram showing how the two recognised forms of TB-IRIS are distinguished from each other.

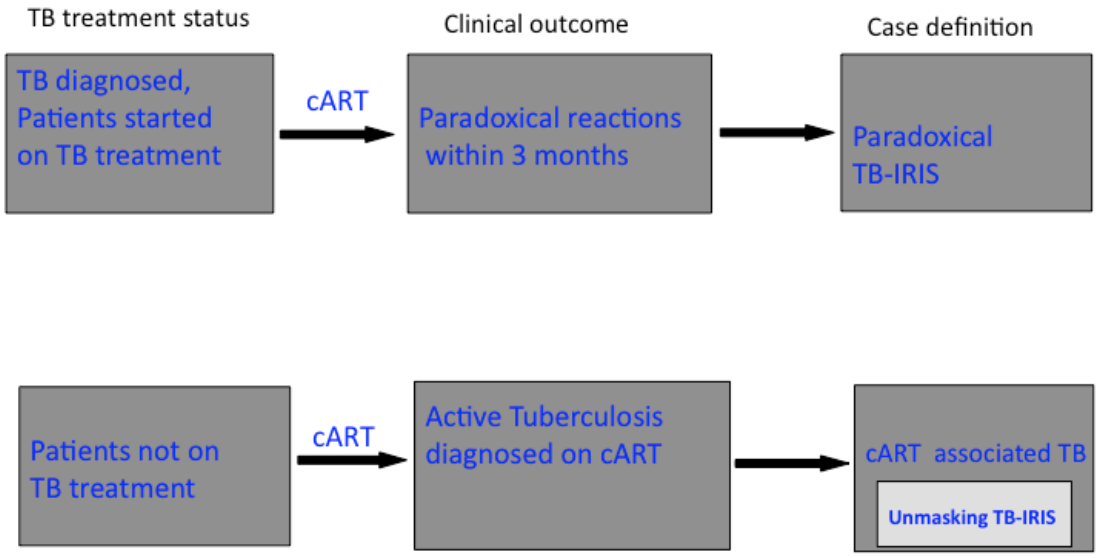


Figure 2 Schematic diagram showing the two categories of TB-IRIS.

Adapted from (Meintjes, Lawn et al. 2008)

1.5.4.1 Paradoxical TB-IRIS

Paradoxical TB-IRIS usually manifests in patients who are diagnosed with tuberculosis prior to initiating cART and are responding well and improving on tuberculosis treatment. Various studies show that the average time of paradoxical TB-IRIS onset after cART initiation ranges from 1-4 weeks (Narita, Ashkin et al. 1998; Breen, Smith et al. 2004; Michailidis, Pozniak et al. 2005). However, incidences of IRIS developing much earlier, in some cases, as early as 3

days after cART commencement while late cases of IRIS, (years after initiation of cART), have also been described (Olalla, Pulido et al. 2002; Huyst, Lynen et al. 2007). On starting cART, these patients develop recurrent, worsening or new symptoms and manifestations of tuberculosis (Lawn, Bekker et al. 2005; Lawn, Myer et al. 2007). Common manifestations include return of symptoms, fever, enlargement of lymph nodes, worsening chest X-rays and pulmonary infiltrates and enlarging effusions (Dhasmana, Dheda et al. 2008). Figure 3 and 4 below illustrate some of the commonly reported features of paradoxical TB-IRIS.

Figure 3 Illustrative case of a female patient with paradoxical TB-IRIS

Figure 3 shows an example of Paradoxical Tuberculosis-associated immune reconstitution inflammatory syndrome lymphadenitis. This young woman was diagnosed with pulmonary TB on the basis of an MTB positive sputum culture that grew Mycobacterium tuberculosis

sensitive to rifampicin and isoniazid and had small neck lymph nodes at that time. Symptomatic response to anti-TB therapy was reported after 3 weeks. She was started on cART at this point at CD4 count of 143 cells/μL. Three weeks latter she developed painful right supraclavicular lymhadenitis with overlying erythema and tenderness.

a

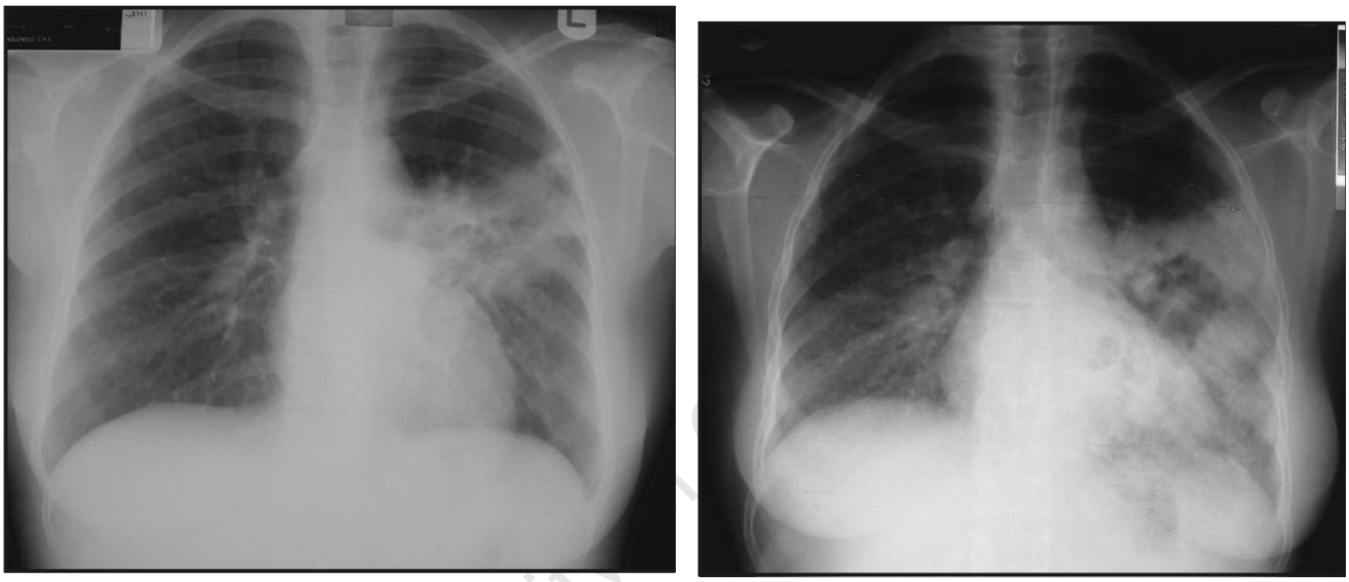


Figure 4a and 4b Worsening chest radiographs in a case of Paradoxical TB-IRIS

Figures 4a and 4b show an example of patient who developed Paradoxical Tuberculosis-associated immune reconstitution syndrome with chest radiograph deterioration. This HIV-infected patient was diagnosed with smear positive pulmonary tuberculosis. Pulmonary infiltrate was noted at that point. She reported an initial symptomatic response to anti-tubercular therapy. She commenced cART at a CD4 count of 63 cells/μL, 3 months after antitubercular therapy. One week later she developed recurrence of her cough and night sweats, consistent with TB-IRIS. Chest radiograph (a) was taken at the onset of TB-IRIS and radiography (b) was taken 10 days later. The upper part of (b) shows an extension of the left-

sided infiltrate with cavitation as well as a new right-sided infiltrate, which was not evident at onset in Figure 4a.

1.5.4.2 Unmasking TB-IRIS

Unlike paradoxical TB-IRIS, the unmasking form of TB-IRIS is less well characterised. Unmasking IRIS typically occurs in patients with unrecognised tuberculosis infection who are not receiving anti-tubercular treatment and on starting cART they manifest with active tuberculosis with unusually accelerated degrees of inflammation (Goldsack, Allen et al. 2003; Breen, Smith et al. 2004; Meintjes, Rabie et al. 2009). The term 'unmasking TB-IRIS' does not refer to all patients who develop tuberculosis while receiving cART. In settings of high tuberculosis incidence, reactivation and re-infection tuberculosis, which may not be related to cART-mediated immune reconstitution, occurs quite commonly in patients on cART. Many cases may also occur because of persistent immunodeficiency. 'Unmasking TB-IRIS' may therefore be classified as a subset of cART-associated tuberculosis (French 2007). This form of IRIS is less well understood and was not the subject of this thesis.

1.6 Risk factors for developing TB-IRIS

Evidence gathered from several studies has identified certain risk factors that predispose patients to developing TB-IRIS, among them are (i) disseminated TB disease or a high antigen load (ii) a low baseline CD4+ count (iii) high baseline viral load and subsequent rapid fall in response to cART (iv) a short interval between anti-tubercular therapy and cART and (v) to a limited extent, genetic factors.

1.6.1 **Disseminated TB disease**

Disseminated and extrapulmonary tuberculosis, which can also be interpreted as higher antigen load has been identified as one of the major risk factors associated with the occurrence of IRIS from various studies (Breton, Duval et al. 2004). In their study of TB-IRIS in Thailand, Manosuthi et al. reported an eight-fold increase in the chance of developing TB-IRIS in patients with extra pulmonary disease (Wendel, Alwood et al. 2001; Manosuthi, Tieu et al. 2009) found higher frequencies of IRIS in patients presenting with both extrapulmonary and pulmonary TB. Several other researchers have since reproduced the findings. This can be explained by the fact that disseminated TB is associated with a higher bacterial load of MTB. HIV-infected TB patients present more often with disseminated tuberculosis, suggesting that the recovering immunity of patients starting cART with underlying TB infection will encounter a higher mycobacterial antigen load. Immune reconstitution inflammatory syndrome to MTB can be seen as a reflection of the interaction between the number and function of appropriate immune cells and the amount of antigen they encounter (Breton, Duval et al. 2004).

1.6.2 **Low-baseline CD4 count**

Advanced immunodeficiency when starting cART has been described as a risk factor for the development of IRIS (French, Lenzo et al. 2000; Michailidis, Pozniak et al. 2005). Several studies have shown that a CD4 count of below 200 cells/µL prior to starting cART is a risk factor for developing IRIS. IRIS has been reported to occur less frequently in patients starting cART with a CD 4 count of greater than 350 cells/µL. A higher degree of immune suppression means that patients are more prone to opportunistic infections and at the same time the homeostatic balance is more advanced in a more damaged immune system (French, Lenzo et al. 2000; Manabe, Breen et al. 2009).

Michailidis *et al.* reported a baseline CD4 count of less than 100 cells/mm3 as a risk factor for TB-IRIS which was also reported by French et al (Michailidis, Pozniak et al. 2005). In other instances the baseline CD4 percentage has been emphasised as a risk factor for TB-IRIS, rather than absolute count (French, Lenzo et al. 2000; Ratnam, Chiu et al. 2006). This may reflect the increased susceptibility of dysregulated immune recovery once HAART is commenced. The significance of the rate of immune recovery and risk of IRIS has had varying reports. French *et al.* reported that the rate of immune recovery measured by increase in CD4 cell count and percentage over 24 weeks had no effect on the risk of IRIS (French, Lenzo et al. 2000), whereas Shelburne *et al.* and Breton *et al.* who focussed exclusively on mycobacterial IRIS, found that CD4 cell percentage increase and CD4:CD8 ratio were factors associated with the development of the syndrome, again raising the issue of an unbalanced immune response in the pathogenesis of IRIS (Shelburne, Hamill et al. 2002; Breton, Duval et al. 2004).

1.6.3 **High baseline viral load and subsequent rapid fall in response to cART**

Being cART naïve and an excellent virologic response (as illustrated by a rapid fall in plasma viral load) has been found to be another risk factor for the development of IRIS in many patients. Patients with a decrease of greater than 2-logs in HIV-1 RNA plasma viral load within 3 months of initiating cART were shown to be more likely to develop IRIS (Shelburne, Hamill et al. 2002; Manabe, Breen et al. 2009). In general, patients lacking a virologic response are unlikely to develop a meaningful immune recovery, and thus are at lower risk of developing IRIS (Boulware, Callens et al. 2008).

1.6.4 **Timing of cART and TB treatment**

A shorter delay between commencing TB treatment and commencing cART has been identified as a significant factor associated with an increased risk of developing IRIS (Breen, Smith et al. 2004; Breton, Duval et al. 2004). This may be explained by the presence of a higher mycobacterial antigen load that the immune system encounters as it is rapidly restored by cART. Various studies have reported that starting cART within 2 months of initiating anti-tubercular treatment is associated with between zero to tenfold increase in the risk of developing IRIS (Shelburne, Hamill et al. 2002; Lawn, Bekker et al. 2005; Lawn, Myer et al. 2007). However, in clinical settings there is a need to balance the risks of opportunistic infections and IRIS when managing patients and considering delaying cART, particularly in the light of recent studies that have shown clear benefits for early initiation of cART in HIV-infected patients (Zolopa, Andersen et al. 2008).

1.6.5 **Genetic factors**

There have been indications that patients may have a predisposition to developing IRIS due to genetic factors affecting the Th1-Th2 balance. To this end some work has been done to investigate whether certain polymorphisms in cytokine genes exacerbate the immunopathological response to various pathogens (Price, Morahan et al. 2002). There were two findings relating to mycobacterial IRIS; firstly, carriage of a C allele at IL-6-174 was common in patients and controls, but was rare in patients with mycobacteria- associated IRIS a (non-carriage of IL-6-174*C). In addition to this, all patients with MAC or MTB associated IRIS were also shown to be homozygous for TNFA-308*1. TNFA-308*1 has been associated with lower production of tumour necrosis factor-α in monocytes, suggesting that it may facilitate IRIS by limiting "TNF-mediated bacteriocidal activity" (Price, Morahan et al. 2002). Genetic polymorphism and racial difference may play a role in IRIS

phenomenon. However, it is important to note that irrespective of these intermittent reports, there has not been a sufficiently large enough study to investigate the effect of genetic predisposition to the development of IRIS and more work still needs to be done on this subject.

1.7 Incidences of TB-IRIS

The reported incidences of TB related IRIS in HIV-infected persons from different populations ranges from 2-43 % (Breton, Duval et al. 2004). Table 2 shows a summary of the various studies and the reported incidences of TB-IRIS from the different studies across the world up to 2010.

Table 2 Summary of reported incidences of TB-IRIS worldwide

Study	Country	Number of Patients with AIDS at enrolment	Proportion of Patients developing TB-IRIS	Mortality from IRIS
Narita et al (1998)	USA	33 (100%)	12 (36%)	-
Wendel et al (2001)	USA	24 (100%)	3 (13%)	-
Navas et al (2002)	Spain	24 (100%)	6 (35%)	-
Breton et al (2004)	France	17 (100%)	16 (43%)	-
Kuramasamy et al (2004)	France	144 (100%)	11 (8%)	-
Michailidis (2005)	UK	55 (100%)	14 (25%)	-
Bourgarit et al (2006)	France	19 (100%)	7 (37%)	-
Chew et al (2006)	Ireland	16 (100%)	4 (25%)	-
Manosuthi et al (2006)	Thailand	167 (100%)	21 (13%)	1%
Elliot et al (2007)	Cambodia	27 (100%)	6 (22%)	4%
Lawn et al (2007)	South Africa	160 (100%)	19 (12%)	1%
Park et al (2007)	South Korea	-	9 (2%)	-
Serra et al (2007)	Brazil	84 (100%)	10 (12%)	-
Eshun-Wilson et al (2009)	South Africa	337 (100%)	56 (17%)	-
Kumarasamy et al (2009)	India	1731 (100%)	95 (5%)	2%
Manosuthi et al (2009)	Thailand	126 (100%)	21 (17%)	-
Breen et al (2004)	UK	28 (100%)	8 (29%)	-
Shelburne et al (2005)	USA	86 (100%)	26 (30%)	-
Michailidis et al (2005)	UK	28 (100%)	9 (32%)	-
Burman et al (2007)	USA	109 (100%)	19 (17%)	-

1.8 Mortality and morbidity due to TB-IRIS

In general, mortality from tuberculosis-associated IRIS is low and is reported very infrequently reported in literature (Table 2). In a systematic review of major IRIS literature, Muller et al have estimated the mortality of TB-related IRIS to be about 3.2% on average (Müller, Wandel et al. 2010). However, morbidity is high and the need for hospitalisation is frequent, resulting in significant draining of resources, particularly in resource-limited settings where the burden is highest. Despite the generally low IRIS-related mortality, of particular note is neurological tuberculosis-associated IRIS, which is particularly associated with poor outcome (Pepper, Rebe et al. 2009).

1.9 Management of TB-IRIS: The use of corticosteroids to treat IRIS

The management and treatment of IRIS remains a grey area with not much consensus and management is often on a case-by-case basis. The use of anti-inflammatory agents such as non-steroidal drugs has been suggested in the treatment of IRIS as these may arrest the acute inflammatory damage during cART initiation in HIV-infected persons (Cheng, Yuen et al. 2000). However, there has not been much clinical evidence published to support their use. Therapeutic aspiration can also be used to temporarily reduce lymph node bulk in cases of massive lympadenitis (Kumarasamy, Chaguturu et al. 2004).

Anecdotal reports on the use of corticosteroids in the treatment of IRIS report evidence of symptomatic improvement without compromising clinical care (Cheng, Yuen et al. 2000; Safdar, Rubocki et al. 2002). In a more recent and comprehensive randomised clinical trial (RCT) Meintjes et al showed that the use of corticosteroid therapy is a potentially useful intervention in treatment and management of paradoxical TB-IRIS (Meintjes, Wilkinson et

al. 2010). Patients were randomised to recive either a 4-week course of prednisone or placebo treatment. Prednisone treatment was shown to reduce (i) days of hospitalisation, (ii) the need for therapeutic procedures and (iii) resulted in more rapid improvements in symptoms, radiography, markers of inflammation such as creatine reactive protein (CRP) as well as performance and quality of life (Meintjes, Wilkinson et al. 2010). However, the use of cortecosteroid should be employed with caution in immuno-suppressed HIV-infected patients.

It is important that a clear diagnosis of paradoxical TB-IRIS be made and antitubercular drug resistance be excluded when considering corticosteroid therapy. The proper screening for symptomatic infection and giving appropriate prophylactic treatment and antimicrobial treatment remains one of the ways of controlling and limiting the frequency of IRIS cases (Cheng, Yuen et al. 2000; Lawn and Wilkinson 2006). It is also important to note that in many cases, paradoxical TB-IRIS is self-limiting, and only rarely is withdrawal of cART recommended as a means of resolving TB-IRIS.

1.10 Immunopathogenesis of TB-IRIS

In many of the cases, the immunopathogenesis of IRIS appears to be pathogen dependent as different types of IRIS seem to have different pathological mechanisms (Lederman 2001). Although data are limited, the inflammatory response associated with IRIS appears to be cell-mediated and antigen-driven (French 2007). As described previously in section 1.6, the development of IRIS generally requires advanced HIV infection and consequently, advanced immuno-suppresion, a rapid response to cART, the presence of inciting antigens and the apparent loss of normal homeostatic control of immune responses, resulting in exaggerated

inflammatory responses (Kestens, Seddiki et al. 2008). It appears that the pathogenesis of IRIS implicates those components that normally maintain homeostasis and modulate the function of the effector cells of the immune system (Munier and Kelleher 2007). Understanding the immunopathogenesis of TB-IRIS will have implications in improved diagnosis and treatment of this syndrome. In this section the various reports and hypotheses that have been put forward on the immunopathogenesis of IRIS are discussed.

1.10.1 The Role of CD4 T cell expansion in TB-IRIS

The majority of paradoxical TB-IRIS cases, particularly paradoxical TB-IRIS, occur in the first 3 months after cART has been initiated or restarted (Meintjes, Lawn et al. 2008). This corresponds to the first phase of immune reconstitution in which there is a rapid increase and redistribution in both the number of circulating CD45RO+ memory cells and CD4 cell function (Autran, Carcelain et al. 1997; Lawn, Myer et al. 2006). The extent of sequestration of activated CD45RO+ memory cells in lymphoid tissue may be directly related to the HIV load with the greatest phase 1 rates of CD4+ cell recovery during cART resulting in increased risk of IRIS (Lederman 2001). In another study, Wilkinson et al also reported significant reductions in viral loads and increases in the numbers of CD4+ T cells as well as expansions in naïve T cells and effector memory cells in HIV-infected, MTB-sensitized patients (Wilkinson, Seldon et al. 2009). Recirculation of this previously sequestered population may provide the opportunity for relevant pathogen specific cells to gain access to sites of infection and engage in the host inflammatory response to foreign antigen. In a more recent study, Antonelli et al reported that IRIS patients had a higher proportion of activated T cells even before ART initiation, including a higher proportion of activated and, so possibly, a dysfunctional Treg suggestive of high antigen load (Mohan, Seaton et al. 2002). In this study IRIS events were reportedly accompanied by high serum levels of IFN-gamma and IL-7,

suggesting a dysregulated effector response to the profound lymphopenia and immunosuppression in these patients. Suggestions on the possibility of IL-7 as a potential biomarker have been put forward based on this work (Schonbeck, Mach et al. 1998), but require further investigation in other patient cohorts to confirm this hypothesis.

1.10.2 **Restoration of the delayed-type hypersensitivity reaction**

HIV infection causes gradual CD4 lymphocytopaenia, along with the inhibition of interleukin-2 mediated lymphocyte activation and proliferation (Boulware and Bohjanen 2007). There is an increase in type 2 cytokine secretion, associated with immunoregulatory mechanisms, which inhibit cell-mediated immune function. This depression of cell-mediated immunity in HIV-infected patients results in non-response to cutaneous PPD, i.e. no skin reaction to the TST even if the patient has had prior exposure to TB. The development of IRIS has been shown to coincide with the restoration of IFN-γ secretion and cell-mediated immune responses to mycobacteria all of which are associated with expansions in the number of antigen-specific T cells leading to restoration of DTH skin test responses to mycobacterial antigens (French and Price 2008; Price, Murdoch et al. 2009). The first indication that the restoration of pathogen specific immunity could lead to immunopathological reactions was observed, when atypical presentations of *Mycobacterium avium* complex (MAC) were seen in patients on zidovudine (ARV) monotherapy (French, Mallal et al. 1992). MAC as an opportunistic infection usually presents as disseminated infection, and is associated with decreased response towards mycobacterial antigens. However, in the case of IRIS, MAC infection presented as localized infection, and was associated with the recovery of a delayed type hypersensitivity reaction to mycobacterial antigens (French, Mallal et al. 1992). Granulomatous inflammation and tissue necrosis are typical of DTH responses (French, Price

et al. 2004). Following these discoveries, other case studies have reported the restoration of a DTH response at the time of TB-IRIS presentation (Narita, Ashkin et al. 1998).

1.10.3 **Antigen-specific responses and TB-IRIS**

While different studies have suggested that antigen-specific responses may be responsible for the development of IRIS, Bourgarit et al were the first group to show evidence of this effect (Bourgarit, Carcelain et al. 2006). In a small prospective trial investigating the immunopathology related to TB-IRIS, HIV and TB co-infected patients initiating anti-TB and cART therapies, enzyme linked immunospot (ELISpot) assays were carried out to quantify interferon-γ secreting Th1 cells specific to various TB antigens (ESAT-6, 85B and PPD). TB-IRIS was shown to be associated with PPD specific TH1 responses with pro-inflammatory events in response to cART, but no significant responses to ESAT-6 and 85B. An increase in the number of PPD- specific cells producing IFN-γ during IRIS in 7 IRIS patients which was not evident in similar control patients who did not develop IRIS was observed (Bourgarit, Carcelain et al. 2006). These data suggested that the acute inflammatory response seen in TB-IRIS patients may be directed against persistent antigens within purified protein derivative. *In vitro* analysis of immunomodulatory cytokines and chemokines in the antigen-stimulated PBMC supernatants of these patients showed high levels of Th1 related cytokines/chemokines but not Th2 cytokines. A larger cross-sectional study by Meintjes et al has since shown that while there is an association between dynamic T-cell expansions and TB-IRIS, similar expansions were also observed in non-IRIS patients (Meintjes, Wilkinson et al. 2008). This study also showed that TB-IRIS is associated with high levels of CRP and confirmed the association with high frequencies of *M.tuberculosis* antigen-specific IFN-γ secreting T cells (Meintjes, Wilkinson et al. 2008).

1.10.4 **Regulatory T cells in TB-IRIS**

In general, regulatory T cells (Tregs) are the main cells responsible for maintenance of a homeostatic state by suppressing other immune cells and preventing host damage from inflammatory responses. Tregs suppress the proliferation of effector CD4+ and CD8+ T cells and suppress their cytokine production, thus limiting the immune response to microbial antigens (Boulware and Bohjanen 2007). Since the balance between allowing clearance of infections and preventing immuopathology is delicate, it was suggested that an unbalanced immune reconstitution of effector and regulatory T cells in patients receiving cART may result in IRIS (Kestens, Seddiki et al. 2008). In a small study comparing a subset of patients who developed *Mycobacterium avium* and *intracellulare* (MAI) IRIS and non-IRIS controls, Seddiki et al showed that IRIS was characterised by the presence of a subset of CD4+ T cells specific to MAI antigen which produced high levels of IFN-γ and IL-2. The study also confirmed an increased proportion of Treg cells in IRIS patients compared with healthy controls, as did another study which observed an increase in Treg at the peak of IRIS symptoms (Tan, Yong et al. 2008; Seddiki, Sasson et al. 2009). Thus the lack of suppression of Tregs may effectively be contributing to IRIS. The high levels of IL-2 seen in these patients may be involved in the survival and maintenance of these Tregs (Fontenot, Rasmussen et al. 2005).

1.10.5 **The role of cytokines and other immunomodulators in TB-IRIS**

There have been suggestions that immunomodulation of interleukins and cytokines may be a possible direction for future treatment of IRIS (Cheng, Yuen et al. 2000), although clinical trials are yet to be done to investigate this. Several cytokines have been implicated in the immunopathology of IRIS although it remains unclear whether an increase in the levels of certain cytokines is a cause or consequence of IRIS. In a study of patients who had a history

of IRIS, Stone et al reported a higher constitutive expression of IL-6 and soluble IL-6 receptor (sIL-6R) in the tissue culture supernatants of patients with a history of IRIS compared to those with no history of IRIS or HIV-negative controls (Stone, Price et al. 2002). The levels of these two proteins were also observed to be higher in mitogen-stimulated and in plasma samples of these patients, although no differences were noted for IFN-γ levels for these patients (Stone, Price et al. 2002). The study by Bourgarit supported the findings of high IL-6 levels in 3 IRIS patients in addition to TNF, IL-1b, IL-10, RANTES and MCP-1 (Bourgarit, Carcelain et al. 2006). In a more recent study Oliver et al have suggested that TB-IRIS is associated with perturbations of the innate immune response to *M.tuberculosis* by showing decreased levels of CCL2 before and after cART and incresead levels of IL-18 and CXCL10. CXCL-10 regulates the trafficking of effector T cells and NK cells to sites of inflammation (Robertson 2002).

1.10.6 **The role of Matrix Metalloproteinases (MMPs) and their inhibitors in TB-IRIS**

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases, which belong to a larger group of metzincin superfamily. Collectively, MMPs are implicated in tissue remodelling processes, chronic inflammatory conditions and are capable of degrading all kinds of extracellular matrix proteins (ECM). More recently they have been found to be involved in multiple mechanisms of immunomodulation (Taylor, Hattle et al. 2006; Sheen, O'Kane et al. 2009). MMPs are also known to be involved in the cleavage of cell surface receptor proteins, processing of bioreactive molecules, chemokine processing, defensin activation, wound healing and have a major role in cell behaviours such as cell-trafficking and leucocyte recruitment (Elkington, O'Kane et al. 2005; Gueders, Foida et al. 2006).

MMPs can be classified broadly by substrate specificity into stromelysins (MMP-3, MMP-10 and MMP-11), collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), gelatinases (MMP-2,

and MMP-9), elastases (MMP-7, MMP-12) and membrane-type MMPs (MT-MMPs, MMP-14, MMP-15, MMP-16 and MMP-17) (Parks, Wilson et al. 2004). However, this classification is artificial as there tends to be overlap in substrate specificity and redundancy of function among many of the MMPs. Table 3 is a summary of the various classes of MMPs and Tissue inhibitors of metalloproteinases (TIMPs) analysed in this study as well as their biological roles and the alternative names. Table 3 shows a summary of the different MMPs and their classifications as well as some common biological functions.

MMP Classification	Gene Name	Alternative name	Biological Function
Collagenases	MMP-1	interstitial collagenase fibroblast collagenase	Breakdown of extracellular matrix Breaks down interstitial collagens type 1, 2 and 3 Expression increased by mechanical force
	MMP-8	neutrophil collagenase collagenase 1	collagen cleaving enzyme in connective tissues of most mammals enzyme is stored in secondary granules within neutrophils is activated by autolytic cleavage Degrades type I, 2 and 3 collagens
	MMP-13	collagenase 3	cleaves type 2 collagen more efficiently than type 1 and 3 may be involved in articular cartilage turnover and cartilage pathophysiology associated with osteoarthritis
Stromelysins	MMP 3	Stromelysin-1 Progelatinase	Degrades fibronectin, laminin, collagens 3, 4, 9 and 10 and cartilage proteoglycans Thought to be involved in wound repair, atherosclerosis, and tumour initiation
	MMP-10	Stromelysin 2	Degrades proteoglycans and fibronectin
	MMP-11	Stromelysin 3	activated intracellularly by furin cleaves alpha-1 proteinase inhibitor, but weakly degrades structural proteins of the ECM
Gelatinases	MMP 2	Gelatinase A neutrophil gelatinase	Degrades type 4 collagen Role in endometrial menstrual breakdown, regulation of vascularisation and the inflammatory response Mutations associated with Torg, Wincester syndrome
	MMP-9	Gelatinase B Type 4 collagenase	degrades type 4 and 5 collagens
Elastases	MMP 7	matrilysin, uterine PUMP	Degrades proteoglycans, fibronectin, casein and elastin Unlike other MMPs, lacks a conserved C-terminal protein domain involved in wound healing and may regulate defensin activity
	MMP-12	macrophage metalloelastase	this protein is thought to be cleaved at both ends to release the active enzyme degrades soluble and insoluble elastin suggested role in the development of emphysema
TIMPs	TIMP1	metalloproteinase inhibitor 1 is a natural inhibitor of the MMPs	a glycoprotein inhibitor expressed from several tissues promotes cell proliferation in some cells may have anti-apoptotic function gene transcription is highly inducible in response to many cytokines and hormones
	TIMP2	metallopreinase inhibitor 2	thought to be a metastasis suppressor inhibitor of MMPs has a unique ability to suppress the proliferation of endothelial cells is critical in maintenance of tissue homeostasis by suppressing the proliferation of quiescent tissues in response to angiogenic factors

Table 3 Summary of the classification of different MMP and TIMP genes and their biological function

(Adapted from Parks et al 2001, Manocone and McGuire 2008, Visse and Nagase 2003, Shapiro and Senior 2009)

In healthy lung, MMPs and their physiological inhibitors, TIMPs are produced in the respiratory tract by a panel of different structural cells and perform multiple roles in normal immune response to infection (Elkington, O'Kane et al. 2005). A strong connection between immune responses and MMP activity has recently emerged and MMPs have been shown to mediate the expression of immunity to infectious pathogens as well as inflammatory processes (Taylor, Hattle et al. 2006). Excessive MMP secretion has been linked with tissue damage in rheumatoid arthritis, pulmonary fibrosis and chronic obstructive pulmonary disease. Host derived MMPs are necessary for the successful eradication of infection which requires the influx of effector cells, killing of pathogen, resolution of inflammation and the remodelling of ECM (Elkington, O'Kane et al. 2005).

As with most enzymes with the potential to damage the host, MMPs are tightly regulated. There are three main regulation mechanisms of MMP synthesis and function:

1. Transcriptional activation of MMPs as they are rarely stored, but requires increased gene transcription to drive secretion.
2. Post-transcriptional processing of MMPs secreted as latent pro-enzymes that undergo proteolytic cleavage for activation.
3. Control of MMP activity by secretion of specific endogenous inhibitors (TIMPs) and α-macroglobulin which bind in a non-covalent manner to the MMPs (Gueders, Foida et al. 2006).

Emerging data suggests a strong association of MMP activity with the various pathological states associated with tuberculosis (Sheen, O'Kane et al. 2009; Elkington, D'Armiento et al. 2011). A number of studies have shown that MMPs, particularly MMP-9 is highly expressed during various manifestations of tuberculosis, including active cavitary and tuberculous

pleuritis. In order to disseminate, MTB must drive host tissue destruction, thus resulting in pulmonary cavitation. MTB disseminates from an initial seeding site to initiate a host response that results in an inflammatory lesion within the lung apex, resulting in cavitation. Once cavitary disease develops, MTB proliferates exponentially, uncontrolled by the immune system and is able to disseminate. The pathogenesis of destructive lung disease caused by tuberculosis remains poorly understood. Evidence of tissue destruction seen frequently in IRIS cases includes abscess formation, suppurative lymphadenitis. Thus, there is evidence to suggest that MMPs may be involved in the immunopathology of TB-IRIS.

1.11 Major Aims of the Study

The main of my study was to characterise the immunopathogenesis of paradoxical HIV-Tuberculosis Immune Reconstitution Inflammatory Syndrome by analysing various biological samples collected from patients who developed paradoxical TB-IRIS and compare these to similar patients who did not develop the condition. The major objectives for this study were:

1. To determine levels of mRNA for selected cytokines, MMP genes and other immune markers in the PBMC of IRIS and non-IRIS patients i.e. *in vitro*.
2. To measure the protein levels of selected cytokines and MMPs secreted in tissue culture supernatants of IRIS and non-IRIS control patients using different immunoassays.
3. To determine the protein levels of the cytokines and MMPs from (1) and (2) above *in vivo* i.e. in serum samples

4. To determine the immune-modulatory effect of prednisone treatment *in vivo* on cytokine and MMP protein levels in sequential samples of IRIS and non-IRIS control patients.

5. To determine the level of MMP activity in biological samples from IRIS patients and non-IRIS controls

2 CHAPTER 2: MATERIALS AND METHODS

The studies presented in this thesis were aimed at characterising the immunopathogenesis of paradoxical HIV-Tuberculosis associated immune reconstitution inflammatory syndrome (HIV-TB-IRIS). In this chapter, detailed descriptions of the clinical enrolment of patients, the various immunological methodologies and a summary of the statistical methods that were employed in these studies are outlined.

2.1 Study sites, clinical recruitment and treatment of patients

Patient assessment, recruitment and collection of the clinical samples was conducted by Professor G. Meintjes and other clinical colleagues. Patients with suspected TB-IRIS were recruited at GF Jooste Hospital a community-based referral hospital, in Cape Town, South Africa, that serves communities with a high prevalence of HIV infection and TB. Patients were also recruited from the Ubuntu Clinic, site B Khayelitsha, Cape Town, South Africa between March 2005 and September 2007 Most patients are commenced on TB treatment and ART in community primary care clinics, but are referred to GF Jooste Hospital when complications occur for investigations and/or admission. New TB cases were treated with 6 months of standard first line therapy consisting of isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) for 2 months followed by HR for 4 months (2HRZE/4HR) while patients with a previous history of TB had streptomycin added to their treatment regimen. All patients were prescribed first line cART combination of stavudine (d4T), lamivudine (3TC), and either efavirenz (EFZ) or nevirapine (NVP). Participants were monitored fortnightly for a period of two months after initiation of cART. Observation during this study was 12 weeks (84 days) after starting cART. Patients who developed paradoxical TB-IRIS were recruited at onset of symptoms while non-IRIS controls were recruited on day 14 of follow-up after commencing

cART. This period was shown to be the median time to the development of IRIS in HIV positive TB patients on cART.

2.2 Inclusion/exclusion criteria and case definitions

Paradoxical-TB-IRIS was defined according to the published and validated International Network of HIV-associated IRIS (INSHI) case definition, which is described in detail in Section 1.5.3. (Chapter 1). Generally, patients included into the study were: cART naive, responded to treatment, had either clinically or microbiologically confirmed tuberculosis in addition to other clinical criteria. In the case of TB-IRIS, patients had to have a clear and confirmed diagnosis of paradoxical TB-IRIS based on a validated case definition and conform to WHO definitions for HIV-associated-tuberculosis. Exclusion criteria included a biological age less than 18 years, known rifampicin-resistant tuberculosis or multi-drug resistant tuberculosis, previous glucorcorticoid therapy, prior cART exposure, life-threatening TB-IRIS and pregnancy among other clinical criteria. A diagnosis of exclusion was conducted to exclude alternative reasons for clinical deterioration of the TB patients.

2.3 Ethical Approval

The University of Cape Town Human Research Ethics Committee approved the studies (REC 337/2004 and 173/2005). All participants enrolled into these studies provided written informed consent.

2.4 Study Designs

Longitudinal and cross-sectional study designs were employed for the analyses presented in this thesis. Immunological analyses were performed on different patient subsets and are described in detail in subsequent section 2.5.

2.4.1 **Cross sectional study**

A cross-sectional study nested within an IRIS cohort was conducted to compare paradoxical TB-IRIS cases and control patients. Patients who were diagnosed with paradoxical TB-IRIS were matched with similar comparable HIV-TB control patients who were treated similarly but did not develop TB-IRIS. The clinical treatment of these patients is outlined in section 2.1. For the case-control cross-sectional analysis, 22 TB-IRIS patient samples (of 32 available) were selected randomly and compared with 22 non-IRIS control patients who participated in a prospective cohort study of 62 HIV-TB patients commencing cART. Selection of the 22 paradoxical TB-IRIS patients and 22 non-IRIS controls was then done in order of sample storage in the freezer provided all samples (supernatnants, RNA and serum) were available for each patient. Patient inclusion criteria into the study included patients who were (1) cART naive, (2) had clinically or microbiologically confirmed tuberculosis or conformed to WHO definitions for HIV-associated TB and responded to treatment (3) in the case of TB-IRIS had a clear and confirmed diagnosis of paradoxical TB-IRIS based on a validated of consensus (INSHI) case definition definition. Patients with confirmed or suspected rifampicin resistance or multi-drug resistant TB were excluded. Cases and controls were prospectively enrolled subject to inclusion and exclusion criteria and the availability of sufficient sample to perform analyses (Figure 5). Observation during this study was 12 weeks (84 days) after starting cART and non-IRIS controls were selected from 28 patients with complete follow-up and sufficient sample who did not develop TB-IRIS (Figure 6). Non-IRIS controls were not individually matched to TB-IRIS cases: instead the range of nadir CD4, days of anti-TB treatment, duration of TB treatment to cART, disease form, age and gender of TB-IRIS cases were used to define similar non-IRIS controls. Baseline viral load was not available in all cases and was therefore not factored. The median interval between starting antiretroviral therapy and the onset of TB-IRIS was 14 days (table 5). The baseline and

clinical charecteristics of these patients are summarised in Section 3.2. and in Table 5. Figure 5 and Figure 6 below show flow diagrams to illustrate the patient recruitment and selection for TB-IRIS and non-IRIS control patients described in the studies presented in Chapters 3, 4 and 5 of this thesis. Results of the cross-sectional analysis are presented in Chapters 3, 4 and 6 of this thesis.

2.4.2 **Randomised Placebo-controlled trial of prednisone for Paradoxical TB-IRIS**

Between 2 June 2005 and 20 December 2008, prior to my involvement in the study, patients were enrolled participants into a randomized placebo-controlled clinical trial of prednisone for the treatment of paradoxical TB-IRIS at G.F. Jooste Hospital in Cape Town, South Africa. Consecutive patients were screened using the standardised INSHI TB-IRIS case definitions. Enrolment was limited to the following four TB-IRIS manifestations to reduce clinical heterogeneity and allow longitudinal radiographic comparison. Only patients with new or recurrent tuberculosis symptoms and at least one of the following TB-IRIS manifestations were enrolled: infiltrate on chest radiograph, enlarging lymph nodes, serous effusion, or cold abscess. If significant clinical deterioration occurred after 2 weeks of follow up, the study protocol allowed participants to be switched to open label prednisone or earlier if life-threatening deterioration occurred. Follow-up was for 12 weeks. Patients were closely monitored during the follow-up period to ensure that cases of TB-IRIS were identified early, thus ensuring compliance and adherence to cART and TB therapy. An increase in CD4 count was also evidence of immunological response, hence compliance to cART in these patients. Additionally, since TB-IRIS is a complication of the two combined therapies, non-adherence to cART and anti-TB therapy would prevent the developmnet of TB-IRIS.

The primary endpoint of this study was cumulative number of days hospitalized and number of outpatient therapeutic procedures performed counted as one additional hospital day. Secondary endpoints of efficacy included symptom score, quality of life score, Karnofsky performance score, chest radiography and C-reactive protein. Patients with immediately life threatening TB-IRIS were excluded. TB diagnosis was made mainly on the basis of culture, smear microscopy or clinico-radiological diagnosis.

Patients were randomised either onto the study drug which consisted of 5mg of prednisone tablets or matching placebo. Participants received study medication of 1.5mg/kg per day for two weeks followed by another 0.75 mg/kg per day for two weeks. Patients were followed up at 1, 2, 3, 4, 8 and 12 weeks and blood samples taken at each time point. Each participant underwent full clinical evaluation and chest radiography. Further investigations were conducted to exclude alternative reasons for clinical deterioration. Patient selection for the subset of RCT patients analysed and presented in this thesis, patient selection was also dependent on availability of a complete set of follow-up sample samples for the immunological analysis. For the analysis presented in Chapters 3 and 4 of this thesis, 16 prednisone treated and 12 placebo treated patients who had sufficient samples (RNA, supernatant and serum) at the 0, 2 and 4 week time-points of follow-up were selected for analysis.

2.4.3 **Longitudinal study**

Consecutive patients with suspected TB-IRIS were evaluated from January 2006 through September 2007 at G.F Jooste, a community-based secondary hospital in Cape Town, South Africa (Meintjes, Wilkinson et al. 2008). Patients were recruited as per the INSHI clinical case definition exclusion using the inclusion criteria described above in section 2.2. Longitudinal analysis was of a prospective cohort of 50 adult patients who started cART

whilst on TB treatment. Participants were prospectively followed-up during the study period and were monitored four times for a period of 12 weeks after initiation of cART. The main clinical outcome was paradoxical TB-IRIS. Eleven patients developed TB-IRIS while 39 did not develop symptoms of TB-IRIS. The 11 patients who developed TB-IRIS were compared to 16 controls who did not develop TB-IRIS under the same conditions for the longitudinal analysis (on the basis of adequate follow-up sample availability). Figure 5 and 6 below show summary flow diagrams of the general patient recruitment of TB-IRIS and non-IRIS control patients for these studies.

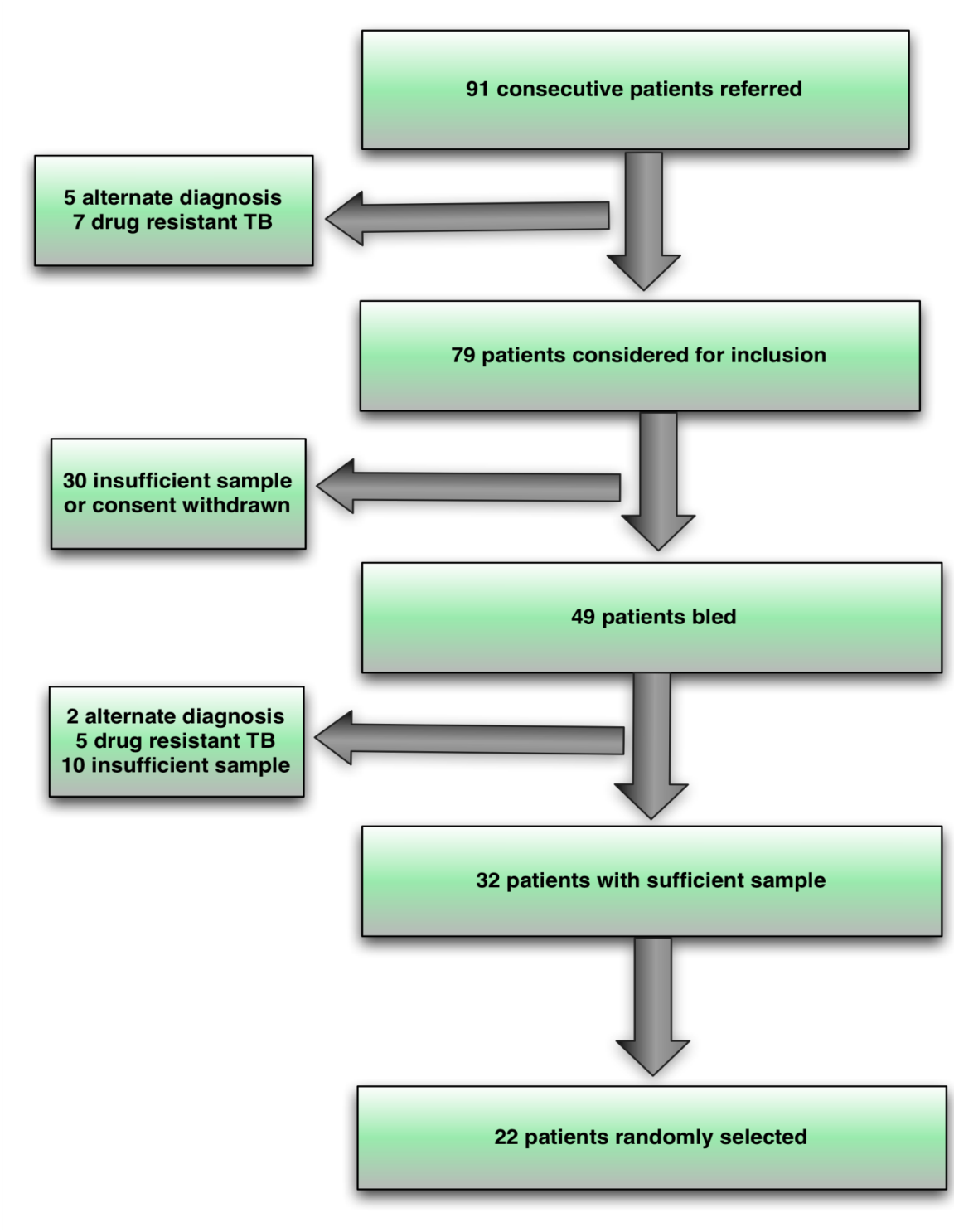


Figure 5 Flow chart summarizing the patient recruitment and selection of TB-IRIS patients.

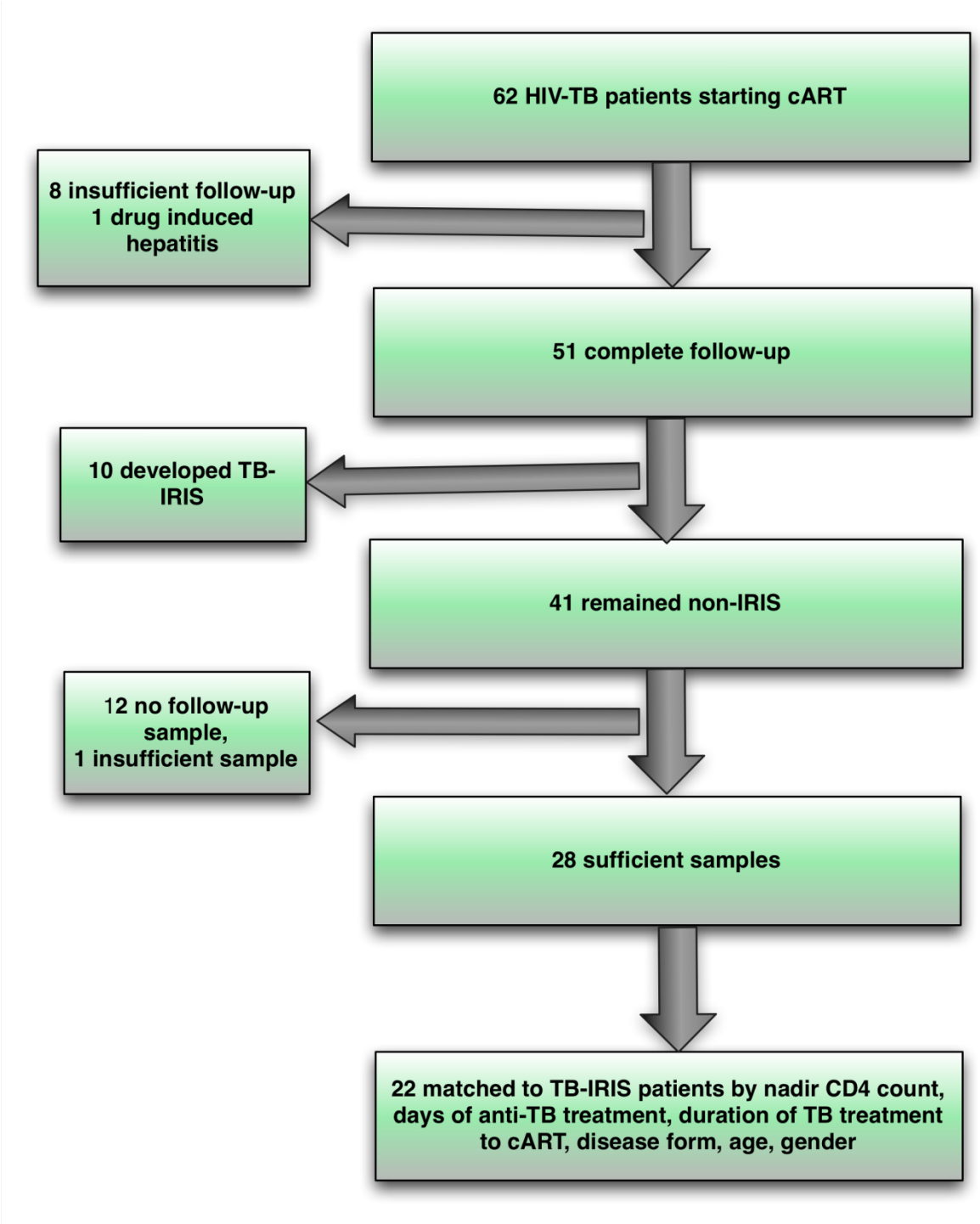


Figure 6 Flow chart showing the patient recruitment and selection of control non TB-IRIS patients.

2.5 Tissue culture protocols and sample cryopreservation

2.5.1 PBMC Isolation

Dr Katalin Wilkinson, Kerryn Matthews, Keira Skolimowska and other colleagues in the Wilkinson lab are acknowledged for the preliminary tissue culture, sample processing and sample cryopreservation that was performed prior to my involvement in this study. The tissue culture work included isolation of PBMC, cell culture, tissue culture supernatants, processing of serum samples and sample cryopreservation. Blood (9mls) was collected by venipuncture into sodium-heparin containing plastic tubes and transported at RT for processing in the lab. Blood was diluted with an equal volume of PBS and carefully layered onto 10ml of Ficoll-Paque in a new 50ml Falcon tube (Ficoll-Paque ^{TM} Plus GE Healthcare, Uppsala, Sweden). The tube was centrifuged for 20 minutes at 700 x g. After this the white layer of cells at the PBS/Ficoll inter-phase was carefully aspirated off using a Pasteur pipette and transferred into a tube containing 15ml of fresh RPMI. Cells were washed twice by centrifuging in RPMI at 600 x g for 10 minutes. After the second wash, cells were resuspended into 10 ml of RPMI. A 10ml aliquot was stained with Trypan blue and cells counted on a haemacytometer to determine viability and numbers. The remainder of the PBMC was then used in the tissue culture protocols as described below.

2.5.2 Cell Culture Experiments

PBMC were plated in RPMI/10% FCS as follows: $5x10^6$/2ml/well in 6 well plates (4 wells) OR $2.5x10^6$/ml/well in 6 well plates (4 wells) OR $2.5x10^6$/1ml/well in 6 well plates (2 wells for the 24 hour time point i.e. RNA& supernatant). For these experiments, we wanted to stimulate the PBMC with the most relevant antigen, a whole bacterium. H37Rv is the best characterised and most widely used laboratory strain of *Mycobacterium tuberculosis* and is a

whole antigen and hence was used in these assays. We hypothesized that the immune response in IRIS would be in response to dead mycobacteria, hence we used heat killed H37Rv. Additionally, the heat killing would make H37Rv non-virulent, allowing the assays to be conducted in a Biosafety Level 2 (P2) laboratory facility. Cells were rested overnight in an incubator and re-stimulated the following morning with Heat Killed H37Rv *Mycobacterium tuberculosis* antigen (kindly supplied by Professor Mark Nicol). Antigen was aliquoted at 5 $X10^7$ and PBMC infected at a multiplicity of infection (MOI) =1 (Bacteria: PBMC). Table 4 shows a summary of how the tissue culture experiments were performed.

Table 4 Summary of Cell Culture protocols

PBMC + HkH37Rv (stimulated)	PBMC- HkH37Rv (unstimulated)	PBMC + HkH37Rv (stimulated)	PBMC- HkH37Rv (unstimulated)
6 hours	6 hours	24 hours	24 hours
Supernatant Discarded PBMC Lysed for RNA	Supernatant Discarded PBMC Lysed for RNA	Supernatant cryopreserved PBMC Lysed for RNA	Supernatant cryopreserved PBMC Lysed for RNA

2.5.3 **Serum isolation procedure**

Serum was harvested from 5mls of blood collected in a yellow top BD vacutainer tube by spinning the blood for 7 minutes and aspirating the serum. Na-azide (10µL of 1% solution)

was added to the serum to make a final concentration of 0.01% before cryopreserving the serum samples at -80°C for further analyses.

2.5.4 **RNA Lysis Procedure**

After 6 and 24 hours of stimulation, cells were lysed in 350µL of RLT-lysing buffer (Qiagen, Valencia, CA) with Beta-Mercaptoethanol added. A cell scrapper was used to dislodge the cells. Cells were aspirated in lysis buffer and then transferred to QIAshredder column (Qiagen, Valencia, CA). The QIAshredder was placed in collecting tubes and the tubes centrifuged at 14 000 rpm for 2 minutes. Tubes were labeled and the lysates stored at -80°C until required for RNA extraction.

2.6 Quantitative RT-PCR

2.6.1 **RNA Extraction Procedure**

RNA was extracted from cryopreserved PBMC lysates (using the RNeasy Kit Mini (Spin) Protocol for Isolation of Total RNA from Animal Cells (Qiagen, Ontario, Canada) as per manufacturer's instructions. About 350µL of 70% ethanol was added to the homogenized lysate and mixed by pipetting up and down. Sample (700µL) was then applied to the RNeasy mini column placed in a 2ml collection tube which was then centrifuged for 15 seconds at 10 000 rpm. Flow-through was discarded before addition of and washing of the column with 700 µL of Buffer RW1. The column was washed twice by spinning with RW1 buffer and the flow through being discarded each time. To ensure complete drying and removal of residual ethanol from the column, after washing with Buffer RPE buffer, the column was spun at full speed to remove excess alcohol. RNA was finally eluted from the column by spinning the column for 1 minute at 10 000 rpm with 30-50 µL of RNase-free water. Eluted mRNA concentration was measured using the NanoDrop^{TM} ND-1000. To attain an RNA working

solution of at least 10ng/µL for each sample mRNA samples were diluted appropriately using RNase-DNase free water.

2.6.2 **Real Time-PCR**

The TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit Protocol (P/N 4392668) from Applied Biosystems was used for RT-PCR according to the manufacturer's instructions (Foster City, California, USA). The reaction mixture was set-up as outlined below: 10µ L of Predesigned Ready to use Assay (PDAR), 1µL of RT-PCR Mix, 0.5µL of TaqMan^{R} RT-Enzyme Mix and 8.5µL of diluted mRNA to make up a reaction volume of 20µL for each sample. Non-template controls were included and the reaction performed in MicroAmp^{R} Optical 96-well reaction plates on an Applied Biosystems sequence detection system (ABI 7000). The following universal PCR thermal cycling conditions were used: reverse transcription Step at 48°C for 15 minutes (hold), enzyme activation step 95°C for 10 minutes (hold), denaturation Step 95°C for 15sec (40 cycles), and annealing/primer extension 60°C for 1 minute (40 cycles).

Normalisation of RT-PCR was performed using Beta Actin primers purchased from the University of Cape Town Molecular and Synthetic DNA Laboratory, while probes were purchased from Applera South Africa (Foster City, California, USA). The Beta Actin assay was performed as per manufacturer's instructions. The sequences of the Beta Actin primers and probes used in these assays are described below:

Beta actin Forward Primer: 5' CCT GGCACCAGCACAAT 3'

Beta actin Reverse Primer: 5' GCCGATCCACACGGAGTACT 3'

Beta actin Probe: 5' ATCAAGATCATTGCTCCTCCTGAGCGC 3' + MGB probes

The reaction was performed by mixing 0.5µL with 0.75 µL of the forward primer and 0.75 µL of the reverse primer together with 8.5µl of diluted mRNA or water (in the non-template

controls) to make up a volume of 20µL for each reaction. The PCR reaction was performed under the same conditions as described below.

2.6.3 **TaqMan^{R} Gene Expression Assays and Assay Details**

Primers and probes were purchased from Applied Biosystems (Applied Biosystems, Foster City, California, USA) as Predesigned ready to used (inventoried) assay reagents in a single formulation tube. Primers with a shorter amplification length were preferred. The following TaqManR gene expression assays were used: Assay ID: IL-1β, Catalogue Number Hs00174097_m1; Assay ID: IL-2, Catalogue Number Hs00174114_m1; Assay ID: IL-4, Catalogue Number Hs00174122_m1; Assay ID: IL-5, Catalogue Number, Hs00174200_m1; Assay ID: IL-6, Catalogue Number Hs00985639_m1; Assay ID: IFN-γ, Hs00174143_m1; Assay ID: TNF-α, Catalogue Number Hs00174128_m1; Assay ID: IL-8, Catalogue Number Hs01038788_m1; Assay ID: IL-10, Catalogue Number Hs00174086_m1; Assay ID: IL-12p40, Catalogue Number Hs01011518_m1; Assay ID: lL-13, Catalogue Number Hs00174379_m1; Assay ID: IL-15, Catalogue Number Hs00542562_m1; Assay ID: IL-17A, Catalogue Number Hs00174383_m1; Assay ID: IL-18, Catalogue Number Hs01038788_m1; Assay ID: IL-19, Catalogue Number Hs00604657_m1; Assay ID: IL-20, Catalogue Number Hs00218888; Assay ID: IL-21, Catalogue Number Hs00222327_m1; Assay ID: IL-22, Catalogue Number Hs00222327_m1; Assay ID: IL-23 , Hs00413259_m1; Assay ID: IL-24, Catalogue Number Hs01114274_m1; Assay ID: IL-26, Hs00218189_m1; Assay ID: IL-27, Catalogue Number Hs00377366_m1; Assay ID: IL-28, Catalogue Number Hs0000820125_m1; Assay ID: IL-29, Catalogue Number Hs00601677_m1; Assay ID: GM-CSF, Catalogue Number Hs00171266_m1; Assay ID: TGF-β1, Catalogue Number Hs00171257_m1; Assay ID: TNF, Catalogue Number Hs00174128_m1; Assay ID: MMP-1: Hs00899658_m1; Assay ID: MMP-2: Hs00234422_m1; Assay ID: MMP-3:

Hs00968308_m1; Assay ID: MMP-7: Hs01042795_m1; Assay ID: MMP-8:Hs01029057_m1; Assay ID: MMP-9: Hs00957555_m1; Assay ID: MMP-10: Hs00233987_m1; Assay ID MMP-11: Hs00968295_m1; Assay ID: MMP-12: Hs00159178_m1, Assay ID: MMP-13: Hs00233992_m1; Assay ID: TIMP-1: Hs99999139_m1; Assay ID TIMP-2: Hs00234278_m1.

2.7 Cytokine Protein Quantification

Luminex XMAP™ technology is a fluorescent bead-based technology based on a combination of existing flow cytometry technology, microspheres (or beads), lasers, digital signal processing and traditional chemistry. Microspheres are dyed internally with red and infrared fluorophores in different ratios. At least 100 different ratios are used to make 100 different bead sets, which allows for the simultaneous detection of multiple analytes in a single sample. Each bead is unique with a spectral signature determined by the red and infrared dye ratio and can be uniquely identified by a classification laser. The immune-complex microsphere is then excited by the classification laser. Luminex is able to identify the specific bead and quantify the specific emission. Luminex kits from different manufactures are commercially available. Detailed descriptions of the kits used in this work are outlined in the following section.

2.7.1 Luminex Analysis of Cytokines

Customized commercial Milliplex™ XMAP kits were purchased from Millipore (Human Cytokine/Chemokine, Catalog Number: MPXHCYTO-60K, MPXHCYTO-60KPMX, St Charles, Missouri, USA). Cytokine levels in 24 hour tissue culture supernatants and serum samples were assessed using 96-well filter plates, on a Bio-Plex platform, Bioplex ™Bio-Rad

100 analyser (Bio-Rad Laboratories, Hercules, USA), according to the manufacturer's instructions. Cytokine assays were performed on an 8-plex panel or 9-plex panels depending on whether the analytes required sample dilution. For IFN-γ, IL-10, IL-12p40, IL-2, IL-15, IL-13, IL-5, IL-4 the assay was performed on 8-plex panels with the samples assayed as neat. Samples were diluted 1:10 before measuring IL-8, IL-1β, MIP-1α, MIP-1β, IL-6, RANTES, TNF-α, GM-CSF, and IP-10 on a 9-plex panel. Antibody-immobilised beads vials containing each of the analytes were vortexed before mixing them and making up the final volume of the bead mix to 3ml with Bead Diluent. Before the assay, the 96-well filter plate was pre-wet by adding assay buffer into each well and incubating the plate. Assay buffer was then vacuumed out before adding 25µL of either standard or control to the appropriate wells. A similar volume of assay buffer was added to the sample wells, followed by 10% FCS RPMI where appropriate (for the diluted 9-plex plate). Sample was added into the appropriate wells, followed by 25µL of pre-mixed beads. Following incubation, excess fluid was removed by vacuum from the plate before washing with wash buffer. Detection antibody was added to each well followed by a further 30 minute incubation of the plate at RT. Without removing the contents, 25µL of Streptavadin-Phycoerytrin conjugate, the reporter molecule, was added to each well to complete the reaction. After incubating for 30 minutes, all contents were vacuumed out before a final wash. Sheath fluid was added to each well to resuspend beads before reading the plate on a Biorad Luminex platform.

2.7.2 **ELISA immuno-Assays**

2.7.2.1 Measurement of IL-17

The quantitative determination of IL-17A protein levels were performed as per manufacturer's recommendations using commercial eBioscience Human ELISA Ready-Set

Go Kit (Catalog numbers: 88-71760). NUNC Maxisorp 96 well plates were coated with 100 µL of capture antibody diluted in coating buffer and incubated overnight. After washing off excess antibody, the wells were blocked with 200µL of assay diluent before the plate was incubated for 1 hour. Standards were prepared by a -fold dilution together with the samples added to the plate, followed by further incubation at RT for 2 hours. Detection antibody was added, followed by diluted Avidin-HRP and 30 minutes of incubation. After incubation, substrate solution was added and the reaction developed by incubating at room temperature. The reaction was stopped with 2M H_2SO_4 stop solution after 15 minutes and OD read at 450nm on a microplate reader. The IL-17A had a sensitivity of 4pg/ml.

2.7.2.2 Measurement of Interferon-γ

Interferon-γ was measured using an in-house developed ELISA protocol using purified anti-human IFN-γ antibody pairs from BD Pharmingen™ (Catalogue number: 551221). A NUNC plate was coated overnight at 4°C with 50 µL per well of capture antibody. PBS-Tween wash buffer was used to wash the plates before blocking with 200 µL PBS/10% FCS and incubating for 2 hours at RT. Standards were serially diluted 1:3, added to the washed plate together with 100µL of sample per well and the plate incubated overnight at 4°C. Following incubation, the plate was washed, 100µL of biotinylated secondary antibody added, incubated at RT for 45 minutes and the excess antibody washed off before addition of diluted Avidin-Peroxidase. After 30 minutes of incubation with OPD substrate solution, the reaction was stopped and OD measured on a plate reader at 490nm.

2.7.2.3 Measurement of TGF-β1

Transforming Growth factor beta-1 (TGF-β1) was measured using antibody pairs from R&D Systems (Catalog Number: DY240) as per manufacturer's recommendations. The samples were not acid activated for this assay. A NUNC ELISA plate was coated with 100µL of

capture antibody diluted in PBS and incubated at RT overnight. After repeated washing of excess antibody with 400µL of wash buffer, 300µL of block buffer was added and the plate incubated for an hour. Following washing, samples and standards were added and the plate incubated for 2 hours. After incubation, 100µL of secondary antibody was added and the plate incubated for 2 hours at RT. After washing off excess antibody, streptavidin-HRP was added and the plate incubated for 20 minutes. Substrate solution was then added and the plate incubated at RT, after which, the reaction was stopped and optical density determined by reading in a microplate reader at 450nm.

2.7.2.4 Measurement of Interleukin-22

IL-22 protein was determined using the Human IL-22 Immunoassay Quantikine kit from R&D Systems (Catalog Number: D2200). Reagents and samples were prepared as per manufacturer's instructions. Wells were pre-wet by adding 100µL of Assay diluent to each well, followed by 100µL of standard or sample to the appropriate wells. After 2 hours of incubation at RT, the plate was repeatedly washed before adding 200µL of conjugate and incubating further for two hours. After washing off excess conjugate enzyme, 200µL of substrate solution was added, incubated for 30 minutes and the reaction stopped as soon as colour had developed. The OD of the plate was read on a microplate reader at 450nm. The assay had a sensitivity of 15.6pg/ml.

2.7.2.5 Measurement of Interleukin-19

IL-19 was determined using the Human IL-19 Immunoassay Quantikine Kit from R&D Systems (Catalog Number: D1900) as per manufacturer's recommendations. Reagents, working standards and samples were prepared as instructed. Prior to the addition of standards, controls and samples, 100µL of assay diluent were added to each well, followed by incubation of the plate at RT for 2 hours. After repeated washing of the plate with 400µL,

200µL of IL-19 conjugate were added to each well. After a 2 hour incubation period at RT, substrate solution was added followed by further 30 minute incubation in the dark with monitoring. Soon after colour development, the reaction was stopped and OD determined on a microplate reader at 450nm. The assay had a sensitivity of 12.2 pg/ml.

2.7.2.6 Measurement of Interleukin-21

IL-21 protein concentration was determined using LEGEND MAX^{TM} Human IL-21 ELISA kit (Catalogue Number: 433807) from BioLegend (San Diego, CA, USA) according to the manufacturer's recommendations. Reagents were left to stabilise to RT and standards prepared as per manufacturer's instructions. The pre-coated ELISA plate was washed and 50 µL of assay buffer added to pre-wet the plate, followed by addition of 50 µL of standards or samples to the appropriate wells. The plate was sealed and incubated for 2 hours at RT with constatnt shaking. Following this, the plate was washed and 100 µL of detection antibody added to each well, followed by a further 1 hour of incubation at RT with constatnt shaking. After repeade washing steps, 100 µL of Avidin HRP D solution was added to each well follwed by 30 minutes of incubation. Substrate solution was added and the reaction plate incubated for 30 minutes in the dark, followed by stopping solution and reading of the OD at 450nm. The sensitivity of this assay was 4.2 pg/ml

2.7.2.7 Measurement of Interleukin-7

IL-7 was analysed using the IL-7 quantikine High sensitivity (HS) assay (Catalogue number HS750) purchased from R&D Systems, (Minneapolis, USA) as per manufacture's instructions. Reagents were brought to RT before being prepared and standards were reconstituted before serial dilutions to produce the standard curve. Fifty µL of assay diluent were added to each well to solubilise the precoated antibody before addition of 200 µL of sample or standards to the appropriate wells. To maximise sensitivity, the plate was incubated

overnight for 20 hours at RT. The plate was then washed thoroughly with 400 µL of wash buffered 6 times, ensuring complete removal of residual buffer by inverting the ELISA plate on dry paper towels in between each wash. After the washing steps, 200 µL of IL-7 conjugate was added into each well before incubating the plate for a further 2 hours at RT, following which the wash steps were repeated. Fifty µL of susbtrate solution were then added to each well and the plate incubated for 45 minutes. After this incubation, amplifier solution was added, followed by a further 45 minute incubation. After colour development, 50 µL of stop solution were added to stop the reaction. The optical density of each well was determined on a microplate reader set at 490 nm. This assay had a sensitivity of 0.6 pg/ml.

2.8 Analysis of Matrix metalloproteinses(MMPs) and their inhibitors

Protein levels for the different MMPs and TIMPs as well as MMP/TIMP complexes in tissue culture supernatants and in serum samples were analysed using Luminex technology and ELISA protocols as outlined in this section.

2.8.1 MMP Multiplex analysis

Luminex assays for MMPs were performed on the BIO-RAD Bioplex 200 System using the Fluorokine MAP Kit Protocol (Catalogue Number LMP001, R&D Systems, Inc) as per manufacturer's recommendations. The reaction plate was prepared by pre-wetting with 100µL/well of wash buffer and then vacuuming out excess liquid. Diluted microparticle mixture (50µL) was added to each well followed by 50µL of sample or standard to each well. The plate was incubated for 2 hours at RT on a microplate shaker. For MMP-1, -2, -3, -7, -8, -12 and -13, samples were assayed undiluted. To measure MMP-9, the tissue culture supernatant was diluted 1:100 and was assayed on a separate plate from the rest of the analytes. The plate was washed three times by adding 100 µL of wash buffer and then

vacuuming out excess liquid. After washing, 50µL of diluted Biotin Antibody cocktail was added to all wells followed by 1 hour incubation at RT. Washing was repeated, followed by addition of Streptavidin-PE to all the wells. The plate was further incubated for 30 minutes before repeating the wash steps. The plate was then read on a Biorad Luminex analyzer within 90 minutes of preparation at both high and low PMT instrument settings. Extra dilutions were included at the lower end of standard curve for low RPMI readings and the machine recalibrated to capture analytes at low concentration. At all times, care was taken to protect microparticles from light.

2.8.2 **Human MMP-10 ELISA**

The concentration of MMP-10 protein in the tissue culture supernatants and serum samples was measured using the Human MMP-10 quantikine kit from R&D Systems (Catalogue number DM1000). This kit measures human pro-MMP10. Reagents, working standards and samples were prepared and diluted as per manufacturer's instruction. Assay diluent (100µL) was added to each well followed by 50µL of standard, control or sample to the appropriate wells. The plate was incubated for 2 hours on a plate shaker followed by washing with 400µL of wash buffer. Following washing, 200µL of MMP-10 conjugate was added to each well and the plate incubated at RT for a further 2 hours. Substrate solution was then added to each well followed by 30-minute incubation for colour development, after which the reaction was stopped and OD read on at 450nm. The assay has an average sensitivity of 4.13 pg/ml.

2.8.3 **Tissue inhibitors of Matrix Metalloproteinases (TIMP) Assays**

Levels of human TIMP-1 and TIMP-2 protein as well as TIMP-2/MMP-9 and TIMP-1/MMP-2 complexes in tissue culture supernatants and serum samples were measured using

Duoset ELISAs from R&D Systems (catalog numbers: DY970, DY971, DY1453, DY1496 respectively). Reagents and samples were prepared and diluted according to the manufacturer's protocol. A 96 well microplate was coated with 100µL of diluted capture antibody and the plate incubated at RT overnight. The plate was washed twice with 400µL of wash buffer before blocking each well with 300µL of reagent diluent. Following a further one-hour of incubation the plate was washed again and this was followed by addition of samples and standards. After another wash step, and incubation with detection antibody for 2 hours, excess antibody was washed off and 100µL of streptavidin-HRP was added to each well. After incubating with substrate solution at RT for 30 minutes, the reaction was stopped and optical density measured on a microplate reader set to 450nm. The TIMP-1 assay had a sensitivity of 11pg/ml while the TIMP-2 assay had a sensitivity of 18pg/ml.

2.8.4 **Gelatin Zymography**

Enzyme activity for MMP-9 was measured using an established gelatinase zymography method. This technique involves the electrophoresis of secreted protease enzymes through discontinous polyacrylamide gels containing enzyme substrate (which can be either type III gelatine or B-casein). After electrophoresis, removal of SDS from the gel by washing in 2.5% Triton X100 solution allows enzymes to renature and degrades the protein substrate. Staining of the gel with commassie blue allows the bands of proteolytic activity to be detected as clear bands of lysis against a blue background.

Tanks and components sufficient for two gels were washed with distilled water and dried before assembly. Reagents and buffers for this assay are summarised in Appendix 2. A gel slab sandwich was constructed and locked into the base unit. The running gel was made and poured with a 25 ml pipette, and overlaid with 1ml of isopropanol at the top. The gel was left

to set for about 20 minutes, after which the isopropanol was poured off, combs inserted and stacking gel poured until overflowing and left to set for 30 minutes. After this, gels were assembled ready to place in tanks and running buffer poured to check for leaks, before placing in a large tank ready to run. Standards and samples were prepared and 20µL loaded into each well. The gel was left to run at 180V for 4 hours until the blue marker ran off the gel. After this, running buffer was poured off and the gel sandwich dismantled. The stacking gel was cut off and a top corner of the gel nipped off for orientation of the gel. The gel was incubated with 200 ml of 2.5% Triton X-100 for 1 hour at RT with gentle shaking to wash off the SDS. Collagenase buffer was then used to rinse and incubate the gel overnight (16 hours) at 37°C. The following day, collagenase buffer was poured off and the gel rinsed in distilled water before incubating it at RT with gentle agitation in staining solution for 2 hours. Staining solution was poured off and rinsed in distilled water before capturing an electronic image of the gel on a Syngene Bio Imaging GelDoc system (Syngene, Hong Kong). Scion image analysis software was used to analyse the images by determining densitometry of the clear bands to measure gelatinase activity of MMP-9.

2.9 Statistical Analysis

The main comparisons were between TB-IRIS and non-IRIS contol patients. Comparisons were also made between prednisone treated and placebo treated TB-IRIS patients from the RCT. Data derived from gene expression studies is not normally distributed. However, logarithmic transformation should make the data more symmetric reducing the effect of outlier values and inter-experimental variations. Based on preliminary studies, typical standard deviations for log-transformed gene expression data from exactly the same genes have a range of 0.4 to 1.4 (unpublished data). Using a sample size of 21 samples in each arm (i.e IRIS vesus non-IRIS) at a 95% confidence interval would give us a conventional power

of 80% , thus making the findings well-powered and generalisable. Based on these statitics, we used a sample size of 22 TB-IRIS cases and 22 non-IRIS controls for the cross sectional analysis.

The delta delta (Δ ΔCT) method used was used for analysis of the RT-PCR data. In this method, Δ CT is calculated by subtracting the Ct of the housekeeping gene from the Ct of the gene of interest. Beta actin was used as the housekeeping gene through out these experiments. The Δ CT of the unstimulated sample was then subtracted from that of the stimulated sample to obtain ΔΔ CT. Fold induction was obtained by adding this value to the mathematical equation $2^{-\Delta\Delta CT}$. This value was then log 10 transformed and used for the fold induction analyses.

GraphPad Prism® Version 5.03 software for windows, and Mac was used for analysis (GraphPad Software, San Diego California USA). Normality of the data was assessed by the D'Agostino & Pearson omnibus normality test. Medians were quoted ± IQR and means ± SD. Paired parametric data was analysed by the student's paired t-test, or repeated measures ANOVA. Paired non-parametric data was analysed by the Wilcoxon signed rank test or Friedman test. Unpaired parametric variables such as comparisons of Delta CT between IRIS and non-IRIS were assessed using the unpaired t-test for parametric data. The Mann Whitney U test was used for analysis of unpaired non-parametric data such as the Delta Ct values between stimulated and unstimulated patients. Comparisons between stimulated and unstimulates IRIS or non-IRIS patients were performed using the Wilcoxon matched-pairs signed rank test. RT-PCR fold induction data was analysed by using unpaired analyses (unpaired t-tests) and the data treate as continous variables. Cytokine and MMP

concentrations were treated as continous variables and were analysed by repeated measures ANOVA and paired t-tests

Randomised-controlled trial (RCT) samples were analysed by one-way ANOVA using the Kruskal Wallis test with no post test correction to accommodate incomplete follow-up data sets. To factor multiple comparisons, p values were multiplied by n-1. Correlations were determined by non-parametric correlation (Spearman correlation) between the selected data sets such as INF-gamma ELISA versus luminex, Delta CT versus cytokine concentration and MMP versus cytokine concentrations.

3 CHAPTER 3: THE ROLE OF PRO- AND ANTI-INFLAMMATORY CYTOKINES IN THE IMMUNOPATHOLOGY OF HIV-TB ASSOCIATED IRIS

3.1 Introduction

Apart from initiation of ART, the causes of immunopathology in IRIS occurrence in patients with tuberculosis remain incompletely understood. Initially recognised in the context of atypical mycobacterial infection and termed immune restoration disease (IRD) (French, Mallal et al. 1992), IRIS in the context of TB is clearly antigen load dependent; its risk factors also include a low nadir CD4 count, high HIV-1 viral load and short interval between commencement of antitubercular and cART therapies (Breen, Smith et al. 2004; Breton, Duval et al. 2004; Shelburne, Visnegarwala et al. 2005; Manosuthi, Kiertiburanakul et al. 2006; Burman, Weis et al. 2007; Lawn, Myer et al. 2007). TB-IRIS presents temporally as two forms. Paradoxical TB-IRIS occurs in patients who are diagnosed with active TB prior to cART, are typically improving on TB treatment and then during early cART develop an immune-mediated paradoxical reaction with new or recurrent clinical and/or radiologic manifestations of TB and the less well defined: unmasking TB-IRIS.

It is important to understand the immunopathogenesis of TB-IRIS in order to inform specific therapies. Past studies on this subject have tended to be either anecdotal or underpowered: particularly important considering the highly heterogenous nature of the condition. To this end, the pathogenesis of TB-IRIS remains ill-understood. Among the early features associated with TB-IRIS is the conversion of a negative TST to strongly positive after ART (Narita, Ashkin et al. 1998). Remarkable (Hengel, Allende et al. 2002) expansions of terminally differentiated tuberculin PPD specific CD4 T cells by flow cytometric analysis in

TST positive patients during ART in the absence of TB-IRIS have also been reported. Further work has documented highly dynamic *M. tuberculosis* antigen specific Th1 T cell expansions are clearly associated with cART mediated immune restoration in TB co-infected persons (Bourgarit, Carcelain et al. 2006; Meintjes, Wilkinson et al. 2008; Elliott, Vohith et al. 2009), although their absence from some patients with TB-IRIS and their presence in similar patients without the syndrome brings into question whether the association is causal (Meintjes, Wilkinson et al. 2008; Elliott, Vohith et al. 2009). One possibility is that such Th1 expansions are associated with defective restoration of regulatory T cell function. However TB-IRIS patients had no difference in the numbers of $CD4^{+}FoxP3^{+}$ positive cells (assumed regulatory) when compared to similar patients who did not develop the syndrome(Meintjes, Wilkinson et al. 2008), an observation subsequently been replicated by others (Tan, Yong et al. 2008; Seddiki, Sasson et al. 2009). TB-IRIS has also been associated with Killer Immunoglobulin receptor (KIR) -negative gamma delta T cells and anti phenolic glycolipid antibodies (Simonney, Dewulf et al. 2008; Bourgarit, Carcelain et al. 2009) and, in a small subset analysis, with a peak of non-specific inflammatory cytokines/chemokines (TNF, IL-6, IL-1Beta, IL-10, RANTES and MCP-1) (Bourgarit, Carcelain et al. 2006). This has lead to the comment that, like H5N1 influenza infection and experimental anti-CD28 therapy, TB-IRIS may be associated with a cytokine release syndrome (de Jong, Simmons et al. 2006; Suntharalingam, Perry et al. 2006).

For this study, I hypothesized that TB-IRIS may be associated with a cytokine release syndrome (Ruhwald and Ravn 2007). To investigate this hypothesis, I performed a study to evaluate the role of pro-and anti-inflammatory cytokines in the pathogenesis of TB-IRS. to investigate this hypothesis in greater depth. To investigate this hypothesis in greater depth, I conducted a case-control study comparing cytokine gene expression and secretion *in vitro*,

and serum cytokine levels *in vivo* for 22 paradoxical TB-IRIS and 22 non-IRIS control patients.

3.2 Results

3.2.1 Summary of baseline characteristics of TB-IRIS and control patients for the cross-sectional study

	TB-IRIS	Non-IRIS	p-value
n	22	22	NA
Median age (Years, IQR)	31 (23.2 - 52.7)	35.75 (22.2 - 54.1)	0.11
Baseline CD4 /µl, IQR	62 (14.0 -193.0)	42.5 (5.0 – 302.0)	0.17
Median days of TB treatment prior to cART	56 (13.0 -186.0)	75.5 (29.0 -173.0)	0.06
Median days of cART to IRIS onset	14 (5.0 -78.0)	14 (14 - 14)	0.94
Female n (%)	68	68	0.99
Previous TB?	8 (36)	3 (14)	0.162
TB disease form n (%)			
Pulmonary or pleural	12 (55)	17 (77)	0.062
Disseminated	7 (32)	4 (18)	0.514
Pericardial	1 (4)	-	-
Lymphadenopathic	2 (14)	1 (4)	0.429
Smear or culture confirmed?	19 (86)	13 (59)	0.045

Table 5 Summary of baseline characteristics of 22 TB-IRIS and control patients Tab

le 5 shows a summary of the baseline and clinical characteristics of the patients analysed for this cross-sectional analysis. For most of the variables analysed, there was no significant difference in the baseline characteristics including age, gender, baseline CD4 count, median days between starting cART and TB treatment. However, TB-IRIS patients tended to have more smear or culture confirmed TB disease (p=0.045) than the non-IRIS controls.

3.2.2 **Transcript abundance of cytokine genes**

Transcript abundance was calculated by subtracting the cycle threshold of beta-Actin from that of the gene of interest to obtain a delta Ct (Δ Ct). At 6 hours the RNA for several genes (e.g. IL-13, IL-15 and IL-17A) in unstimulated cells from non-IRIS patients tended to be slightly but significantly higher than TB-IRIS (Table 6). Stimulation with MTB increased the abundance of all transcripts studied in both groups with the exception of TGF-b. After Bonferroni (n-1, 15) correction of multiple comparisons, the abundance of IL-17A was significantly greater in stimulated TB-IRIS cultures. At 24 hours the RNA for IL-5 in unstimulated cells from non-IRIS patients was significantly higher than TB-IRIS and conversely the levels of IL-2, IL-15 and TNF higher in TB-IRIS (Table 7). Stimulation with MTB increased the abundance of all transcripts studied in both groups with the exception of IL-18 and TGF-b (whose level significantly decreased in non-IRIS). The abundance of IL-1b, IL-5, IL-6, IL-10, IL-13, IL-17A, IFN-g, GM-CSF and TNF were significantly greater in stimulated TB-IRIS cultures.

Table 6 Delta CT values for cytokine genes after 6 hours of *in vitro* culture in the presence or absence of heat killed *M. tuberculosis*

	Unstimulated					Stimulated					unstimulated vs. stimulated	
mRNA	IRIS	IQR	non-IRIS	IQR	p	IRIS	IQR	non-IRIS	IQR	p	IRIS	non-IRIS
IL-1β	5.9	4.4-6.9	5.0	3.5-6.6	0.193	-2.6	-2.9-1.4	-1.2	-2.1-0.2	0.01	<0.001	<0.001
IL-2	14.7	13.9-16.0	14.5	13.9-15.1	0.474	9.3	7.4-11.5	12.2	9.5-13.6	0.005	<0.001	<0.001
IL-4	16.5	15.5-17.8	15.9	14.6-17.2	0.136	15.0	13.9-16.7	15.8	14.8-16.8	0.17	<0.001	0.889
IL-5	ND		ND		NA	ND		ND		NA	NA	NA
IL-6	12.1	10.4-14.1	10.1	8.4-12.2	0.01	2.6	1.8-3.4	3.5	2.0-5.2	0.08	<0.001	0.001
IL-8	2.9	1.4-4.3	2.2	0.9-2.2	0.53	-1.5	-3.2-0.0	-0.6	-2.9-1.8	0.49	<0.001	0.02
IL-10	7.2	6.4-8.2	6.6	5.8-7.6	0.04	5.9	3.4-7.1	6.1	5.0-7.5	0.25	0.003	0.27
IL-12p40	16.4	15.2-17.4	14.7	13.1-16.4	0.007	10.7	9.1-11.8	11.6	9.8-13.2	0.25	<0.001	<0.001
IL-13	18.8	17.2-20.3	16.4	14.7-18.5	<0.001	12.3	11.1-13.4	14.1	13-15.6	0.009	<0.001	0.004
IL-15	10.5	10.0-11.4	9.8	9.2-10.2	<0.001	7.8	7.2-8.8	8.3	7.8-9.5	0.09	<0.001	0.003
IL-17A	19.8	18.7-20.8	17.6	15.4-19.0	0.001	13.7	12.2-15.7	16.3	14.4-19.0	0.003	<0.001	0.506
IL-18	18.2	14.6-20.0	17.5	13.7-19.0	0.21	14.8	11.2-18.3	12.2	9.7-14.4	0.03	0.003	0.002
IFN-γ	10.4	9.6-11.8	9.7	9.3-10.8	0.039	5.3	4.2-6.7	7.5	6.1-8.0	0.004	<0.001	<0.001
GM-CSF	13.9	12.7-15.1	13.5	11.7-14.5	0.163	4.8	2.7-6.7	6.9	4.6-9.4	0.038	<0.001	0.001
TGF-β	3.7	3.2-4.1	3.3	3.1-3.6	0.03	3.5	2.9-4.0	3.3	3.1-3.9	0.71	0.17	0.61
TNF	7.8	7.3-8.3	7.7	7.1-8.3	0.5	2.2	1.0-2.2	3.6	2.5-4.9	0.008	<0.001	<0.001

ND = Not detected, NA = not applicable. p values are uncorrected for multiple comparisons in the table but multiple comparisons we factored in the analysis (see text)

Table 7 Delta CT values for cytokine genes after 24 hours of *in vitro* culture in the presence or absence of heat killed *M. tuberculosis*

	Unstimulated					Stimulated					unstimulated vs. stimulated	
mRNA	IRIS	IQR	non-IRIS	IQR	p	IRIS	IQR	non-IRIS	IQR	p	IRIS	non-IRIS
IL-1β	6.4	-1.3-7.8	6.7	4.0-8.6	0.202	-2.7	-3.6-1.7	-0.6	-1.9-0.2	<0.001	< 0.001	<0.001
IL-2	11.5	9.0-12.9	12.7	12.2-13.7	0.014	8.3	7.5-9.4	10.0	7.0-12.7	0.129	< 0.001	<0.001
IL-4	15.5	14.9-16.7	16.1	15.3-17.1	0.409	14.4	14.1-15.8	15.4	14.0-16.6	0.382	0.001	0.004
IL-5	20.2	19.3-20.6	18.9	18.0-20.0	0.003	12.1	11.4-14.7	17.0	13.9-20.1	0.001	< 0.001	0.13
IL-6	12.0	4.0-13.8	12.4	9.7-13.9	0.78	1.3	0.3-3.7	4.6	3.1-5.9	<0.001	< 0.001	<0.001
IL-8	3.0	-0.2-3.8	2.6	0.4-5.1	0.48	-3.7	-4.6-2.1	-1.5	-2.8-1.6	0.006	< 0.001	0.002
IL-10	6.9	5.1-8.1	6.9	6.0-7.1	0.74	4.9	3.1-6.4	6.8	5.9-8.5	<0.001	< 0.001	1,0
IL-12p40	16.2	12.1-17.5	16.9	13.9-18.1	0.20	8.1	6.5-9.4	10.0	7.8-13.3	0.005	< 0.001	<0.001
IL-13	17.6	13.5-10.3	17.6	14.8-18.4	0.61	8.8	7.9-10.0	11.0	9.4-13.7	0.002	< 0.001	<0.001
IL-15	9.6	8.6-10.5	10.6	10.1-11.4	0.005	8.4	7.9-8.7	8.6	8.0-9.6	0.14	0.004	<0.001
IL-17A	18.3	16.3-19.5	18.2	17.7-19.4	0.698	12.7	11.5-14.1	13.9	12.8-15.3	0.048	< 0.001	<0.001
IL-18	9.9	9.2-10.4	10.5	9.0-12.6	0.14	9.9	8.9-10.7	9.6	8.6-11.4	0.85	0.45	0.36
IFN-γ	9.4	6.0-10.6	9.6	8.6-10.7	0.259	3.3	1.8-5.1	6.8	4.3-8.1	0.002	< 0.001	<0.001
GM-CSF	11.8	8.6-14.3	13.6	10.8-15.0	0.382	2.8	-0.9-3.7	5.0	3.9-6.8	<0.001	< 0.001	<0.001
TGF-β	4.7	4.4-5.4	4.5	4.1-4.9	0.1	4.6	4.3-4.9	4.8	4.5-5.6	0.16	0.18	0.004
TNF	7.3	2.7-7.90	8.2	7.7-8.5	<0.001	1.4	1.1-2.0	3.2	2.4-4.9	<0.001	< 0.001	0.002

3.2.3 **Fold induction analysis of Cytokine genes**

The fold induction of genes was calculated by the Delta delta Ct method and values normalised by $\log_{10}$ transformation (Figure 7). In TB-IRIS patients at 6 hours IL-1b, IL-5, IL-6 and GM-CSF were more than 100 fold induced and IL-2, IL-8, IL-12p40, IL-13, IL-17A, IFN-g and TNF more than 10-fold. Gene induction was higher in TB-IRIS than in non-IRIS controls for IL-1b, IL-2, IL-4, IL-6, IL-10, IL-13, IL-15, IL-17A, IFN-gamma, GM-CSF and TNF ($p \leq 0.05$). Bonferroni correction of p values indicated the differences in IL-6 (mean log difference 0.78 ± 0.23, $p_{corr} = 0.033$), IL-12p40 (mean log difference 0.81 ± 0.26, $p_{corr} = 0.047$), IL-13 (mean log difference 1.16 ± 0.23, $p_{corr} < 0.01$), IL-17A (mean log difference 1.08 ± 0.33, $p_{corr} = 0.041$) and IFN-g (mean log difference 0.78 ± 0.21, $p_{corr} = 0.011$) to be most significant. At 24 hours the fold induction in TB-IRIS patients (with the exception of IL-2, IL-15 and IL-18 which showed a reduction) tended to be similar to the 6-hour time point. However fewer differences between TB-IRIS and non-IRIS were observed due to increases that occurred between 6 and 24 hours in the latter group. Significant differences between TB-IRIS and non-IRIS controls persisted, however for IL-8 (mean log difference 0.64 ± 0.24, p = 0.011), IL-10 (mean log difference 0.74 ± 0.18, p = 0.002), IL-15 (mean log difference -0.29 ± 0.12, p = 0.021) and TGF-b1 (mean log difference 0.36 ± 0.12, p = 0.006). TGF-b1 tended to be minimally influenced by the presence of MTB and all fold values were close to baseline.

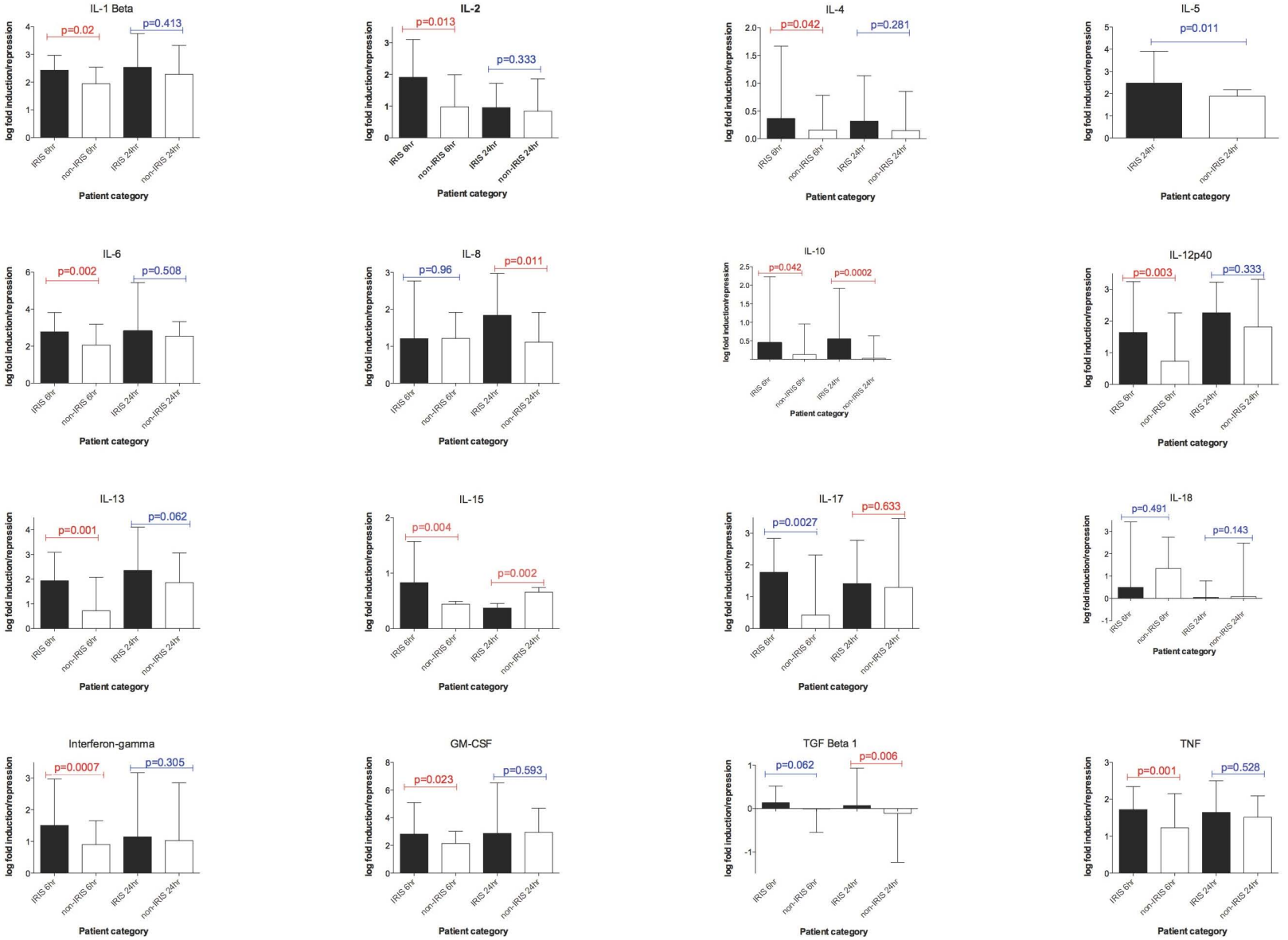


Figure 7 Average log fold induction of cytokine genes by heat killed *M. tuberculosis* in TB-IRIS and non-IRIS patients

3.2.4 **Cytokines secreted into cell culture supernatant**

The corresponding tissue culture supernatants for 20 of the IRIS and 19 non-IRIS patients were assayed for cytokine content by multiplex analysis. Beads for TGF-Beta and IL-18 were unavailable. IL-5 and IL-15 protein were undetectable except in small quantities in 3 samples in the case of IL-5. IL-4 and IL-17, whose levels were close to lower detection limits, did not differ between TB-IRIS and non-IRIS (Figure 8). Otherwise levels were consistently and significantly higher in TB-IRIS. After correction of p-values for multiple comparisons the largest and significant fold differences were in IL-12p40 (~ 40-fold: 40 pg/ml, IQR 4-300 versus 0, IQR 0-16, $p_{corr} = 0.01$), IL-1b (22-fold: 2616 pg/ml, IQR 399-4788 versus 118, IQR 23-240, $p_{corr} = 0.001$), GM-CSF (9.6-fold 721 pg/ml, IQR 254-6129 versus 75, IQR 45-170, $p_{corr} = 0.001$), TNF (8.6-fold 6.79 ng/ml, IQR 4.62-12.67 versus 0.78, IQR 0.25-1.41, $p_{corr} = 0.001$), IL-10 (6.4-fold 876 pg/ml, IQR 267-3070 versus 137, IQR 75-287, $p_{corr} = 0.004$), IL-6 (6.3-fold 17.7 ng/ml, IQR 5.64-34.25 versus 2.81, IQR 0.73-5.56, $p_{corr} = 0.01$), IL-2 (3.5-fold 283 pg/ml, IQR 100-898 versus 80, IQR 9-128, $p_{corr} = 0.04$) and IL-8 (1.4-fold 133.4 ng/ml, IQR 117.8-144.0 versus 96.1, IQR 65.7-107.6, $p_{corr} = 0.001$). Of these cytokines only IL-2 is exclusively of lymphoid origin, the others being predominantly the products of myeloid cells.

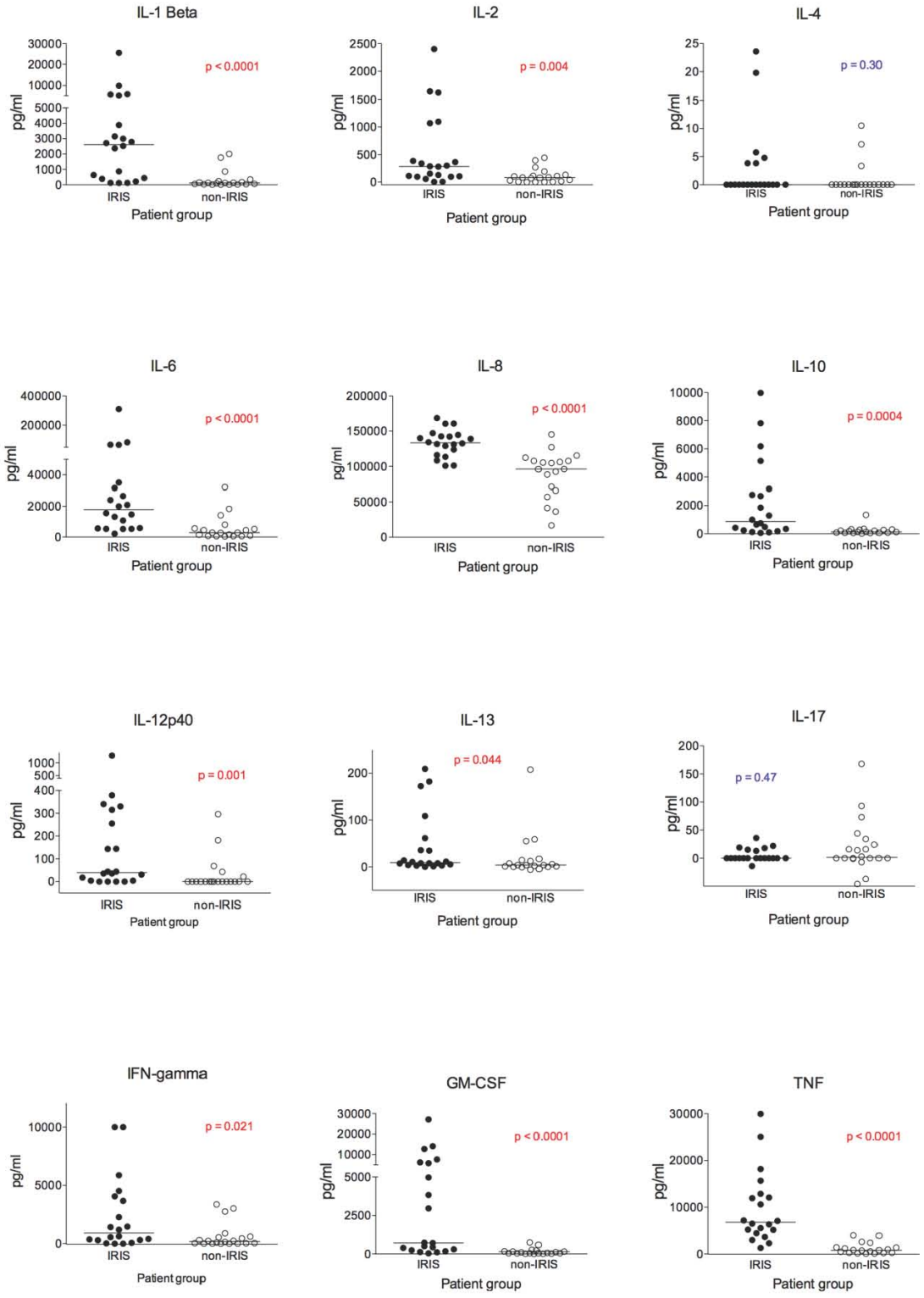


Figure 8 Cytokine content of tissue culture supernatants for the most differentially upregulated cytokine genes

3.2.5 **Correlation of Transcript Abundance versus Cytokine Concentrations in TB-IRIS and non-IRIS patients**

To investigate the relationship between the mRNA levels or transcript abundance and the secreted cytokine concentration in the corresponding tissue culture supernatants, delta CT values (6 and 24 hours for TB-IRIS and non-IRIS patients) were correlated with cytokine concentrations. Table 8 shows a summary of the correlations of the Delta Ct versus protein concentrations i.e. is Spearman correlation (r) and p-values. The lower the delta Ct value the more abundant the transcript is; hence the negative values of the Spearman r values indicate a negative correlation with lower delta Ct values (more abundant transcript) expressing higher concentrations of cytokines. In general, there was a good correlation between transcript levels and cytokine concentration for many of the cytokines particularly for both 6 and 24-hour cultures after MTB-stimulation (Table 8). For IL-1b, IL-2, IL-4, IL-6, IL-10, IFN-gamma, IP-10, IL-13 and GM-CSF there was a negative spearman correlation coefficient and significant p-values ($p \leq 0.05$) in the stimulated 6 and 24-hour cultures. However, IL-15 transcript did not correlate well with tissue culture supernatant protein secretion as show by the p value, which was not statistically significant for both 6 and 24 hours.

Table 8 Spearman Correlation and p-values of Cytokine gene expression versus secreted cytokine concentrations

	unstim 6hrs		unstim 24 hrs		stim 6hrs		stim 24 hrs	
	spearman r	p-value	spearman r	p-value	spearman r	p-value	spearman r	p-value
IL-1b	-0.435	0.006	-0.246	0.131	-0.593	<0.0001	-0.797	<0.0001
IL-2	-0.173	0.292	-0.231	0.158	-0.894	<0.0001	-0.648	<0.0001
IL-4	-0.274	0.092	0.043	0.793	-3.810	0.017	-0.337	0.036
IL-6	-0.525	0.001	-0.199	0.224	-0.599	<0.0001	-0.839	<0.0001
IL-8	-0.310	0.055	-0.171	0.298	-0.308	0.056	-0.552	0.003
IL-10	-0.282	0.082	-0.529	0.001	-0.625	<0.0001	-0.798	<0.0001
IL-12	0.118	0.481	-0.331	0.039	-0.568	0.0002	-0.275	0.090
IL-15	-0.249	0.126	0.029	0.860	-0.112	0.496	-0.147	0.371
IFN-g	0.090	0.587	0.304	0.060	-0.746	<0.0001	-0.722	<0.0001
IP-10	-0.429	0.006	-0.477	0.002	-0.528	0.001	-0.700	<0.0001
TNF	0.117	0.480	0.015	0.928	-0.780	<0.0001	-0.884	<0.0001
IL-13	0.020	0.902	0.229	0.161	-0.519	0.001	-0.731	<0.0001
GM-CSF	-	-	-0.771	<0.0001	-	-	-0.848	<0.0001

3.2.6 **Internal validity of IFN-gamma determination by luminex determination**

Luminex analysis is a convenient and powerful technology but has on occasions been reported to correlate poorly with ELISA (arbitrarily defined as gold standard). There was insufficient sample to secondarily test all analytes. However the same supernatant and cells were additionally assayed for IFN-g secretion by both ELISA and ELISpot using the same stimulus, MTB H37Rv. Correlation between ELISA and Luminex values for IFN-gamma was very strong as shown in Figure 9 (Spearman r = 0.701, $p < 0.0001$). However, Luminex analysis consistently rendered higher values.

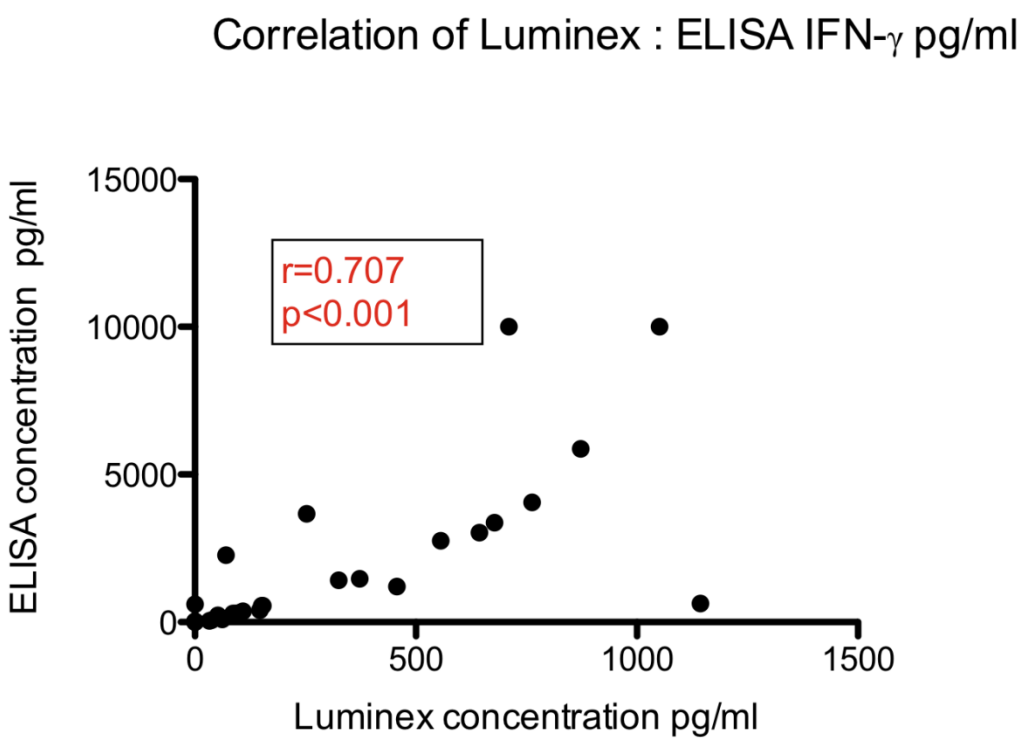


Figure 9 Correlation between ELISA and Luminex analysis for IFN-gamma in tissue culture supernatants

3.2.7 **Cytokine concentrations in serum samples**

Based upon the quantitative RT-PCR and supernatant results we next assayed the level of the most consistently discriminatory cytokines in serum samples taken from the same patients at the same time i.e. IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p40, IL-13, TNF, IFN-γ, IP-10 and GM-CSF. Serum concentrations of IL-1b, IL-2, IL-13 and GM-CSF were consistently close to the lower limit of assay detection and did not significantly differ between TB-IRIS and Non-IRIS groups. The serum concentrations of IL-6, IL-8, IL-10, IL-12p40, IFN-g and TNF were significantly higher in the serum of TB-IRIS patients (Figure 10). After correction of p-values for multiple comparisons (multiplied by n-1), the largest and significant fold differences were in TNF (3.3-fold 90 pg/ml, IQR 17-156 versus 27, IQR 10-45, $p_{corr} = 0.002$), IL-6 (7.3-fold 44 pg/ml, IQR 22-64 versus 6, IQR 0-21, $p_{corr} = 0.001$), and IFN-g (~ 22-fold 22 pg/ml, IQR 10-79 versus 0, IQR 0-8, $p_{corr} = 0.02$). Next, I stratified the IRIS patients on the basis of severity of IRIS. Most TB-IRIS patients in this study had either disseminated TB or evidence of multiple organ involvement (Table 5). However four of these patients were classified as having clinically localized (usually lymphadenopathic) disease, which may be regarded as less severe IRIS. In these patients a clear trend towards lower serum cytokine levels was seen with 22/24 (92%, 95%CL 74-97%) cytokine values falling on or below the median (shown as grey circles in Figure 10). Thus the elevation or otherwise of cytokines appears to relate partially to TB-IRIS severity. However, it is important to note that this study was underpowered to make adequate conclusions on the association of TB-IRIS severity with cytokine levels. Future studies could be performed to further explore this association.

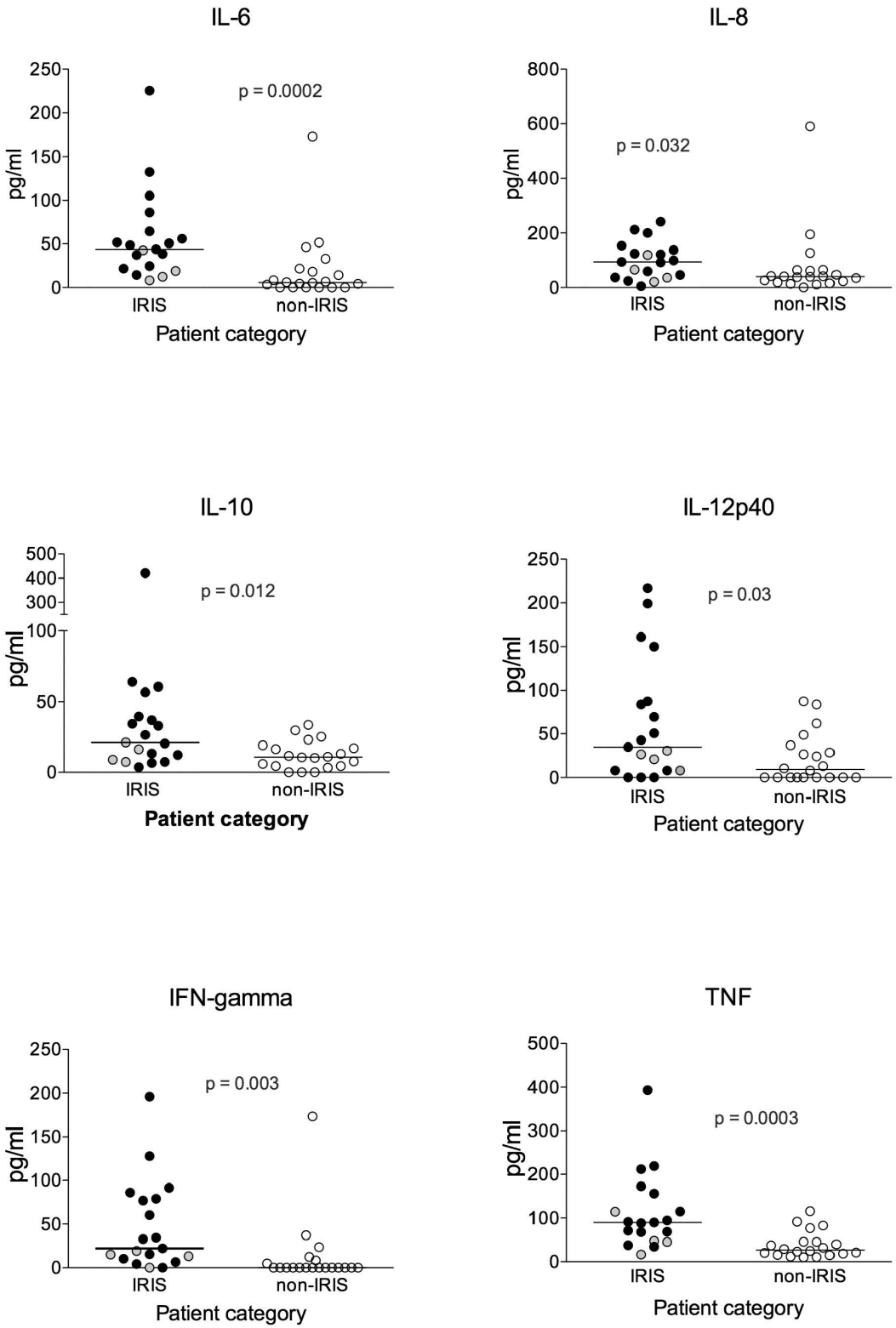


Figure 10 Serum cytokine levels in IRIS and non-IRIS control patients for the most consistently discriminatory cytokines

3.2.8 **Analysis of IL-7 in TB-IRIS and non-IRIS control patients**

Recent studies have reported higher serum IL-7 levels and suggested that IL-7 could be used as a biomarker of IRIS. To investigate the role of IL-7 in TB-IRIS, I analysed mRNA expression by quantitative RT-PCR in a subset of TB-IRIS and appropriate control patients. IL-7 protein expression was also measure in the plasma samples of TB-IRIS and control non-IRIS patients. IL-7 mRNA expression was generally very low in both patient groups and did not show any distinct differences between TB-IRIS and non-IRIS patients. Similarly there was no significant difference noted between IRIS and non-IRIS patients over a 3-week period of follow-up (Figure 11). The median levels of IL-7 were 10.7, 10.1 and 8.3 pg/ml at week 0 (cART commencement), week 2 (IRIS onset time) and week 3 respectively. In the control arm, the median levels of IL-7 were 11.6, 12.6 and 10.5 pg/ml respectively at these same time points. Thus in these patients, although high levels of IL-7 were expressed, there was no significant difference in the levels of this cytokine between IRIS and non-IRIS patients.

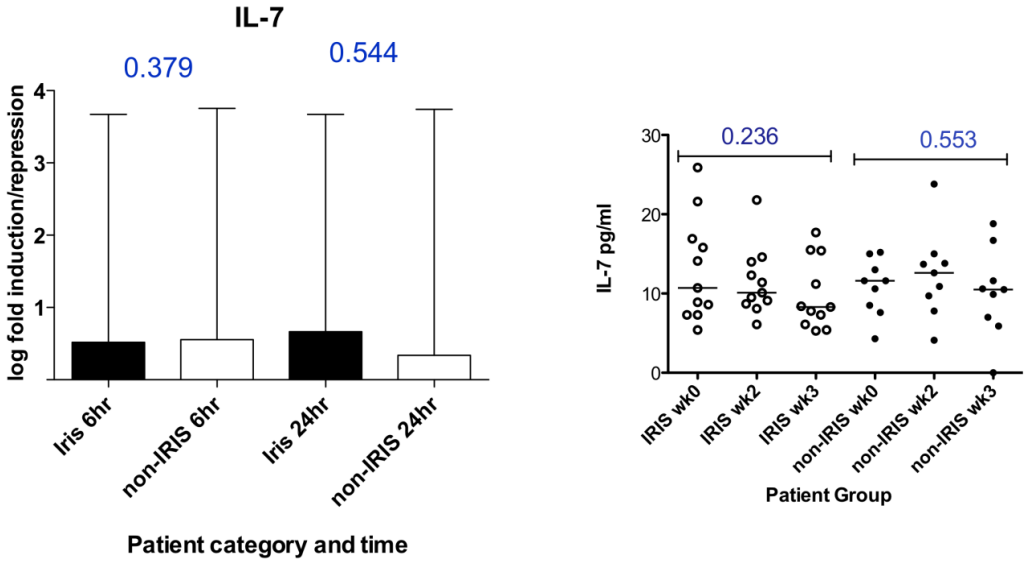


Figure 11 Log fold induction and IL-7 concentrations in TB-IRIS and non-IRIS control patients

3.2.9 **Effect of cortecosteroid therapy on cytokine concentrations in serum samples**

Expert opinion favours the use of adjunctive corticosteroid therapy in some cases of TB-IRIS, an opinion recently provided greater evidence by the randomized placebo-controlled trial of prednisone in TB-IRIS that showed this therapy was associated with more rapid resolution of symptoms (Meintjes, Wilkinson et al. 2010). To better explore cause and effect, I analysed serum cytokine levels in a subset of 10 TB-IRIS trial participants who were randomised to receive corticosteroid therapy (1.5mg/kg daily for 2 weeks then 0.75 mg/kg for a further 2 weeks) or placebo treatment for four weeks. The levels of IL-6 (week zero 40 pg/ml IQR 19-51, week 2 11 pg/ml IQR 8-13, week 4 10 pg/ml IQR 6-22, p = 0.006) and TNF (week zero 144 pg/ml IQR 63-219, week 2 40 pg/ml IQR 24-92, week 4 29 pg/ml IQR 24-101, p = 0.038) significantly declined whereas no effect on IFN-g levels was observed (Figure 12). Figure 12 shows the changes in the concentrations of IL-6, IL-10 and TNF (pg/ml) in serum samples for 10 TB-IRIS patients who were prednisone-treated and were longitudinally followed up for 4 weeks.

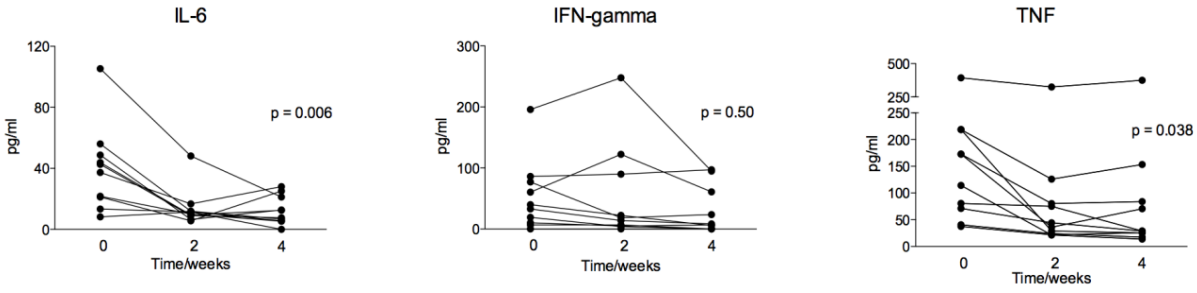


Figure 12 Longitudinal follow-up of prednisone treatment on serum cytokine levels in TB-IRIS patients

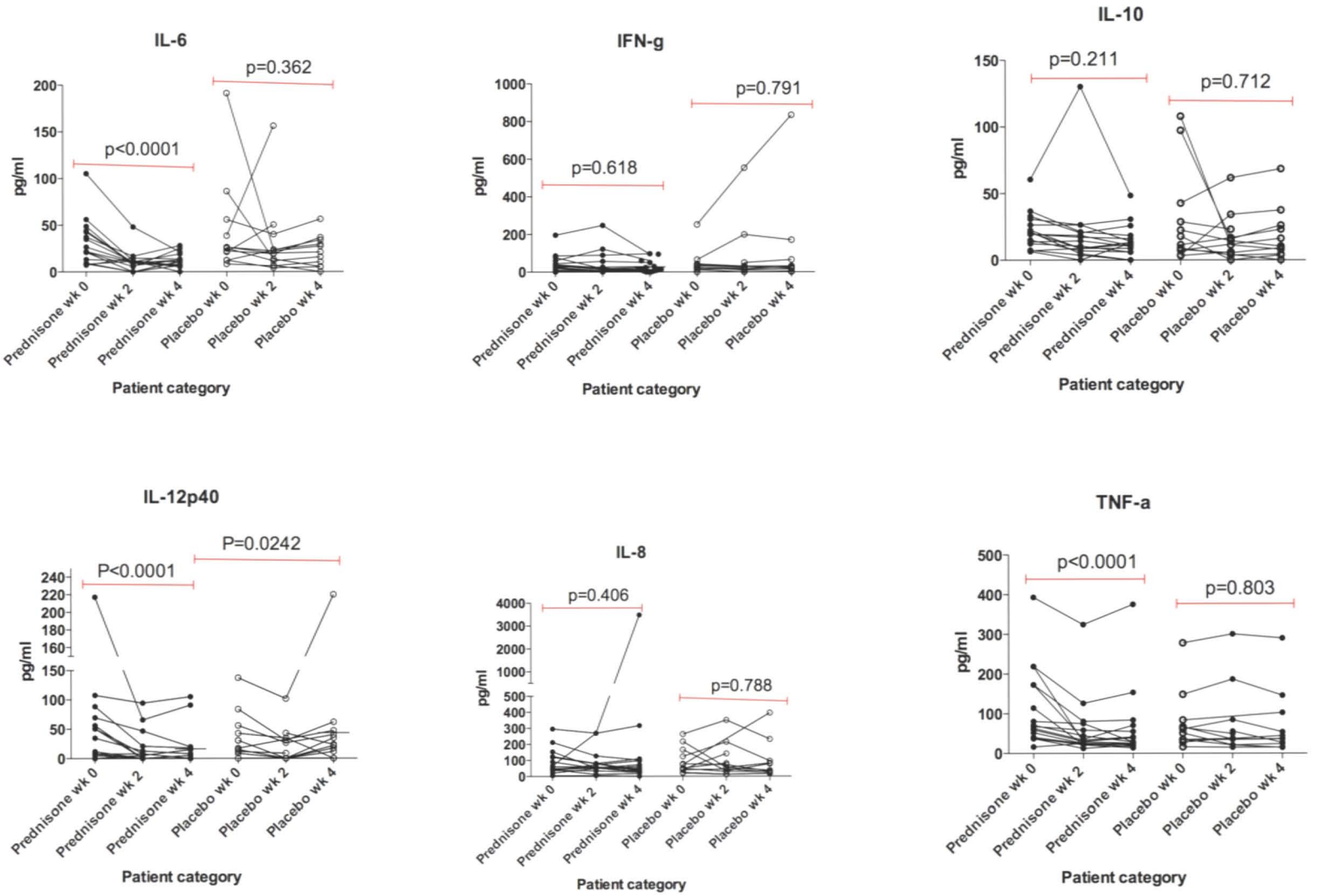


Figure 13 Comparison of prednisone vs placebo treatment on serum cytokine levels in TB-IRIS patients

Figure 13 shows the comparison of cytokine levels between prednisone and placebo treated patients for IL-6, IFN-gamma, IL-10, IL-12p40, IL-8 and TNF. While there was a significant decrease in the levels of IL-6, IL-12p40 and TNF in the prednisone treated patients, there was no change in the levels of these cytokines over the same period in the placebo treated patients. For IL-8, IL-10 and IFN-gamma, there was no difference between prednisone and placebo treated patients, just like in the longitudinally followed-up patients.

3.2.1 Summary of analyses of cytokines in TB-IRIS

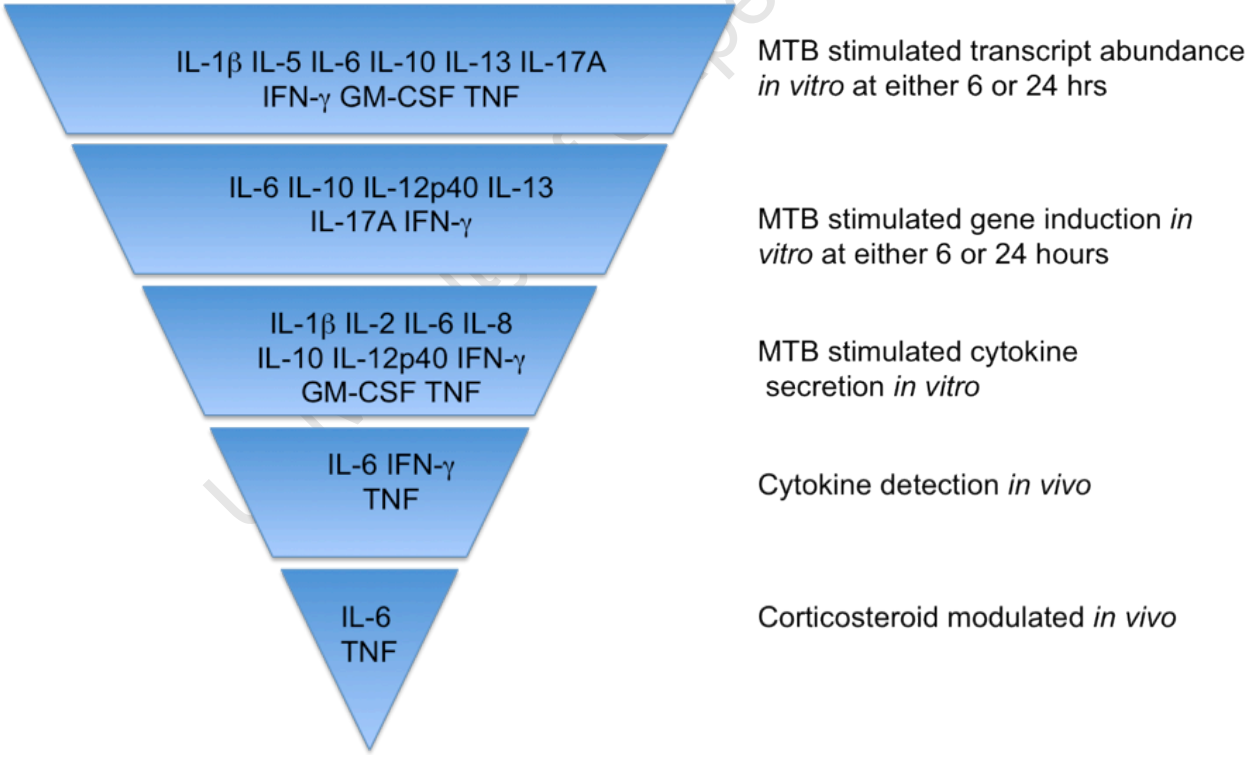


Figure 14 Summary of pro- and anti-inflammatory cytokines that were differentially higher in TB-IRIS

3.3 Discussion

Stimulation of PBMC with *M. tuberculosis* increased the abundance of the majority of transcripts with IL-1Beta, IL-5, IL-6, IL-10, IL-13, IL-17A, IFN-g, GM-CSF and TNF being significantly greater in stimulated TB-IRIS cultures at either the 6 or 24-hour time points. MTB-stimulated gene induction *in vitro* was significantly greater in TB-IRIS patients for IL-6, IL-10, IL-12p40, IL-13, IL-17A and IFN-γ. In tissue culture supernatants, the levels of IL-12p40, IL-1Beta, IL-2, IL-6, IL-8, IL-10, IL-12p40, IFN-γ, GM-CSF and TNF were higher in TB-IRIS patients. In serum samples, significantly higher levels in TB-IRIS patients were observed for TNF, IL-6, and IFN-gamma and the serum levels of IL-6 and TNF decreased during prednisone therapy of TB-IRIS. Thus many pro- and anti-inflammatory cytokine transcript and protein levels are elevated in TB-IRIS patients, strongly suggesting that cytokine release contributes to pathology and symptoms in this condition. IL-6 and TNF were elevated under all conditions and decreased in serum during corticosteroid therapy such that blockade of these cytokines may be a novel and rational approach to immunomodulation in TB-IRIS.

The observations that higher levels of cytokines were consistently detected by luminex versus by ELISA indicate that luminex could be a potentially more sensitive technique than ELISA. The assay detection limits were much lower for luminex, and so lower levels of protein, which may not have been detected by standard ELISA. These observations are in agreement with findings previously published on the evaluation of different commercial bead-based kits, where higher levels of the assessed cytokines were detected by commercial bead-based methods as compared to ELISA (Djoba Siawaya, Roberts et al. 2008)

However, TB-IRIS patients tended to have more smear or culture confirmed TB disease (p=0.045) than the non-IRIS controls. These observations are consistent with the recent findings by Day et al (Day, Abrahams et al. 2011) which reported progressive changes and associations between *Mycobacterium tuberculosis*-specific T cell functional capacity and mycobacterial load in the context of tuberculosis infection and disease. Thus the sputum load in the TB-IRIS patients who tended to have smear or culture positive TB in these patients may have had a confounding as these patients were likely to have more polyfunctional T cell subsets, and hence produce more cytokines. However, while ESAT-6 and CFP-10 antigens were used to restimulate the PBMC, for the work presented in this thesis, cells were restimulated by heat killed H37Rv *M.tb.*

The cytokine release syndrome (sometimes referred to as a cytokine storm or hypercytokinaemia) occurs in a number of infectious and non-infectious diseases including graft versus host disease (GVHD) (Holler, Kolb et al. 1990), acute respiratory distress syndrome (ARDS) (Belperio, Keane et al. 2006), sepsis (Lappin and Ferguson 2009), H5N1 influenza (de Jong, Simmons et al. 2006) and the systemic inflammatory response syndrome (SIRS) (Lenz, Franklin et al. 2007). The experimental drug TGN1412 also caused serious acute illness likely to be driven by cytokines when given to six participants in a Phase I trial (Suntharalingam, Perry et al. 2006). The syndrome does not appear to have a quantitative definition but is characterised as including fever, hypotension, increased endothelial permeability, oedema and vasodilatation. TB-IRIS can be of acute onset especially the unmasking form and fatal ARDS as has been previously described (Goldsack, Allen et al. 2003; Lawn, Wilkinson et al. 2008; Lawn, Wainwright et al. 2009). Patients with paradoxical TB-IRIS frequently have prolonged fever and tachycardia and are at increased risk of venous thromboembolism (Meintjes, Rangaka et al. 2009; Pepper, Rebe et al. 2009). Whilst the

levels of serum cytokines observed in the current study are not as high as those reported in the TGN1412 trial for example or in H5N1, the exaggerated cytokine responses we observed in TB-IRIS patients compared to similar patients who do not experience the syndrome may contribute substantially to pathogenesis.

Anti-retroviral therapy effectively suppresses HIV-1 replication and thereby allows recovery of CD4 numbers and function with the most rapid CD4 rise occurring early in therapy. It is thus logical to investigate whether dysregulated CD4 responses contribute to TB-IRIS. Very large antigen specific Th1 CD4 expansions accompany cART mediated immune restoration in both TB-IRIS patients and similar co-infected persons who do not develop the syndrome (Bourgarit, Carcelain et al. 2006; Meintjes, Wilkinson et al. 2008). In keeping with these observations an increase in IFN-gamma transcript abundance, fold induction and both secreted and serum cytokine in TB-IRIS patients was observed (Tables 6 and 7 and Figures 7). Increased IL-12p40 fold induction and secreted cytokine and also increased IL-2 in tissue culture supernatants from TB-IRIS patients were also observed. However these observations suggest that other lymphocyte subsets other than Th1 may also contribute. Thus transcript from the 'Th2' genes, IL-5 and IL-13 were elevated in TB-IRIS patients although that did not translate into protein differences.

It is also interesting to note that the transcript of IL-17A was, after correction for multiple comparisons, the only significantly elevated in TB-IRIS patients at 6 hours; a difference reflected at 24 hours and in fold induction. However, IL-17A protein levels were low and did not differ between groups. IL-17 is markedly pro-inflammatory and has been implicated in the early protective immune response to tuberculosis (Khader, Bell et al. 2007). In humans IL-17 secretion in response to tuberculosis appears mediated by a distinct T cell subset with

phenotypic characteristics of long-lived central memory cells (Scriba, Kalsdorf et al. 2008). The lack of protein secretion we observed might have been due to IFN-gamma mediated suppression of IL-17 in vitro (Harrington, Hatton et al. 2005). IL-17 is indirectly chemotactic for neutrophils (Laan, Cui et al. 1999) and the cold abscesses that occur in TB-IRIS are characterised by the presence of neutrophils. Future work on the potential for early IL-17 production to contribute to inflammation in TB-IRIS may therefore be of interest.

IL-6 and TNF secretion was identified as elevated in all circumstances and potentially amenable to immune modulation, which is consistent with the finding that polymorphism in these genes may associate with the risk of IRIS (Price, Morahan et al. 2002). IL-6 is a key driver of the acute phase response and we have previously documented that the C-reactive protein (CRP) is invariably elevated at presentation of TB-IRIS (Meintjes, Rangaka et al. 2009). Thus blockade of IL-6 or TNF may be a rational approach to immunomodulation in this condition.

TNF has consistently been associated with both protection and pathology in tuberculosis (Bekker, Maartens et al. 1998; Keane, Gershon et al. 2001; Wilkinson, DesJardin et al. 2001). Recent case reports have documented a beneficial effect of TNF blockade on paradoxically deteriorating TB in HIV-1 uninfected patients (Blackmore, Manning et al. 2008; Wallis, van Vuuren et al. 2009). However a clinical study of anti-TNF antibodies in TB-IRIS would face a number of difficult issues because it is recognised this therapy has a prolonged half-life and also leads to the reactivation of tuberculosis (Keane, Gershon et al. 2001).

4 CHAPTER 4: MATRIX METALLOPROTEINASES INVOLVEMENT IN THE TISSUE DAMAGE ASSOCIATED WITH HIV-TB IRIS

4.1 Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of degrading all components of the extracellular matrix at neutral pH including fibrillar type I collagen, a key structural fibril which is otherwise highly resistant to enzymatic degradation (Parks and Shapiro 2001). The lung matrix is supported by highly stable fibrillar collagens (Brinckerhoff and Matrisian 2002; Elkington, O'Kane et al. 2005; Elkington, D'Armiento et al. 2011). MMPs re known to perform tissue remodelling, repair and modulation of immune responses (Park, Hwang et al. 2005; Gueders, Foida et al. 2006; Taylor, Hattle et al. 2006; Sheen, O'Kane et al. 2009). MMPs are rarely expressed in healthy tissues while their biological activity is highly regulated (Chang, Wysock et al. 1996; Shapiro 1998). Excessive MMP production has been reported in diverse inflammatory conditions such cancer, Chronic Obstructive Pulmonary Disease (COPD), sarcoidosis, interstitial lung disease, arthritis and atherosclerosis (Shapiro and Senior 1999; Manicone and McGuire 2008).

Emerging data suggests an association of MMP activity with pathology in various states of tuberculosis. Advanced pulmonary tuberculosis is associated with a locally destructive process of cavitation which plays an important part in transmission of disease whose pathogenesis remains incompletely understood (Elkington, O'Kane et al. 2005; Taylor, Hattle et al. 2006; Friedland 2008; Elkington, D'Armiento et al. 2011). TB-IRIS is a cART and immune-mediated deterioration which occurs in patients diagnosed with tuberculosis who are responding to anti-TB treatment but develop subsequent clinical deterioration on cART

(Dhasmana, Dheda et al. 2008). Paradoxical TB-IRIS is the best-characterised form of this condition. Characteristic features of the condition include swelling and suppurative lymph nodes, chest-x-ray infiltration with cavitations, weight loss and formation of tissue abscess (Meintjes, Lawn et al. 2008). Central to these features is immune-mediated tissue destruction and breakdown of the normal extracellular matrix structure.

Advanced pulmonary tuberculosis is associated with a locally destructive process of cavitation which plays an important part in transmission of disease (Elkington, O'Kane et al. 2005; Taylor, Hattle et al. 2006; Friedland 2008; Elkington, D'Armiento et al. 2011). Recent work by Volkman et al has demonstrated that disruption of MMP-9 function attenuated granuloma formation and bacterial growth, suggesting that targeting of MMP-9 production could be a promising host-targeting tuberculosis therapy (Volkman, Pozos et al. 2010). TB-IRIS is associated with a tissue degrading phenotype. Little is known about the immunopathology of tissue damage in TB-IRIS. Matrix metalloproteinases (MMPs) have the unique ability to degrade components of the Extracellular Matrix (ECM). A tissue degrading phenotype is evident in TB-IRIS. I hypothesized that MMPs may play a role in tissue degradation in HIV-associated TB-IRIS. MMP gene expression and concentrations of secreted protein (*in vitro*) were analyzed in *M.tuberculosis* stimulated PBMC cultures from TB-IRIS controls. To investigate the role of these tissue-degrading enzymes in the immunopathogenesis of TB-IRIS, I analyzed MMP gene expression and protein secretion in paradoxical TB-IRIS participants and similar controls that did not develop TB-IRIS.

4.2 Results

4.2.1 Baseline characteristics of Study Participants

The baseline characteristics of the patients used for this cross-sectional analysis were previously described in Table 5. Twenty-two TB-IRIS and 22 non-IRIS participants were included in the analysis. There were no significant differences in age, gender and baseline CD4 counts between the two groups. Baseline viral loads were not routinely measured in this study. The median time from starting TB treatment and that to cART commencement, reporting of TB-IRIS and sample collection was similar in both groups of participants (Table 5). However, a greater proportion (86%) of participants in the TB-IRIS compared to 59% of the non-TB-IRIS control group had either culture or smear confirmed TB disease (p =0.045).

A second group of participants originated from a randomized placebo-controlled trial of prednisone (RCT) of TB-IRIS patients which has been reported previously (Meintjes, Wilkinson et al. 2010). Table 9 is a summary of these baseline and clinical characteristics for the subset of RCT patients analysed in this study. There were no significant differences in gender or baseline CD4 count between the prednisone and placebo treated groups. The proportion of participants with previous TB infection, extrapulmonary TB, and IRIS manifestation were also similar between the two groups. However, a difference of marginal statistical significance in the median days of TB treatment prior to cART between the placebo-treated and prednisone treated group (p=0.04) was observed.

Table 9 Baseline characteristics of TB-IRIS prednisone vs. placebo treated patients

	Prednisone treated	Placebo treated	p-value
n	16	12	NA
Female n (%)	10 (63)	5 (42)	0.45
Baseline CD4/μL	48 [13-181]	68.5 [5-156]	0.67
Median days of TB treatment prior to cART	67 [22-180]	30 [13-115]	0.04
Median days of cART to IRIS onset	14 [5-23]	7 [3-32]	0.08
Previous TB? (%)	3 (19)	2 (17)	1.00
Basis of TB Diagnosis			0.71
Culture confirmed	9	8	0.71
Smear only	4	2	0.67
Clinical TB	3	2	1.00
TB Disease Form			
Pulmonary only	9	4	0.28
Extrapulmonary	7	8	0.44
IRIS manifestation n (%)			
New Lymph nodes	6 (38)	3 (25)	0.69

4.2.2 **Transcript abundance of MMP genes**

Transcript abundance of MMP genes in the PBMC cultures of 22 TB-IRIS and 22 non-IRIS control patients was assessed after 6 and 24 hours of stimulation with *M.tuberculosis* (shown in Tables 10, table 11 and table 12). Table 10 shows a summary of the effect of *M.tuberculosis* stimulation in either TB-IRIS patients or controls at 6 and 24 hours. In general, stimulation of PBMC increased the transcript abundance for multiple MMPs in both the TB-IRIS and non-IRIS groups. In particular, MMP-1, and MMP-10 transcripts increased on stimulation in the TB-IRIS and control groups at both 6 and 24 hour time points while MMP-7 transcript increased in the TB-IRIS group only. MMP-2, MMP-11 and TIMP-2 transcripts were down regulated on stimulation in both patient groups.

When TB-IRIS participants were compared with controls in the unstimulated samples, MMP-3, -7, -10 transcripts were more abundant in non-IRIS controls ($p \leq 0.01$). A six-hour stimulation did not result in any significant difference in the transcript levels between TB-IRIS and controls (Table 11). In the 24-hour cultures, MMP-1, MMP-7, MM-10 and TIMP-1 transcripts were significantly higher in the TB-IRIS compared to controls (Table 12) ($p \leq 0.02$). MMP-12 transcript was higher in controls in the unstimulated 24-hour cultures. Thus, stimulation of PBMC with *M.tuberculosis* clearly increased transcript levels for multiple MMP genes, particularly at the 24-hour time point.

Table 10 Comparison of the differences in transcript abundance between heat killed *M. tuberculosis* stimulated and unstimulated IRIS and non-IRIS PBMC cultures

	Median 6 hour delta CT values						Median 24 hour delta CT values					
mRNA	unstim IRIS	stim IRIS	**p-value**	unstim non-IRIS	stim non-IRIS	**p-value**	unstim IRIS	stim IRIS	**p-value**	unstim non-IRIS	stim non-IRIS	**p-value**
MMP-1	15.0	10.2	< 0.001	14.8	10.3	< 0.001	15.6	5.6	< 0.001	15.4	9.8	<0.001
MMP-2	7.5	8.0	0.07	7.7	7.4	0.41	7.2	9.4	< 0.001	6.3	9.4	<0.001
MMP-3	20.3	15.9	< 0.001	18.8	17.3	0.06	19.0	20.1	0.56	18.7	17.2	0.81
MMP-7	10.3	9.1	0.002	9.2	9.3	0.72	9.4	6.8	< 0.001	9.3	8.7	0.05
MMP-8	19.8	18.8	0.19	18.9	19.3	0.63	19.0	19.5	0.08	18.9	17.2	0.88
MMP-9	2.5	1.7	0.14	1.3	1.4	1.00	2.8	0.3	< 0.001	2.3	0.7	<0.001
MMP-10	17.9	11.3	< 0.001	16.3	13.5	0.004	18.3	10.5	< 0.001	18.1	13.4	<0.001
MMP-11	14.4	18.5	< 0.001	13.7	19.0	< 0.001	18.5	16.0	0.93	15.5	19.3	0.14
MMP-12	19.8	19.3	0.82	18.9	19.0	0.63	20.2	13.8	< 0.001	18.7	14.9	0.002
TIMP-1	2.7	2.2	0.02	2.8	2.6	0.30	3.0	2.6	0.13	3.5	3.4	0.68
TIMP-2	2.6	4.0	< 0.001	2.5	3.7	< 0.001	2.3	3.7	0.04	2.1	3.7	<0.001

Table 11 Median Transcript abundance as measured by delta CT values for MMP genes after 6 hours of *in vitro* culture in the presence or absence of heat killed *M. Tuberculosis.*

Median 6 hour delta CT values

	unstimulated					**stimulated**				
mRNA	IRIS	IQR	non-IRIS	IQR	**p-value**	IRIS	IQR	non-IRIS	IQR	**p-value**
MMP-1	15.0	12.9-18.5	14.8	11.6-17.9	0.50	10.2	6.7-12.2	10.3	8.6-12.9	0.33
MMP-2	7.5	6.9-8.1	7.7	6.46-8.50	0.72	8.0	7.2-8.9	7.4	7.1-9.3	0.51
MMP-3	20.3	19.3-20.8	18.8	16.8-19.3	<0.001	15.9	14.2-19.0	17.3	14.9-18.8	0.49
MMP-7	10.3	9.1-11.1	9.2	8.1-10.2	0.01	9.1	7.9-10.1	9.3	7.8-10.9	0.75
MMP-8	19.8	17.8-20.8	18.9	17.2-19.4	0.14	18.8	11.5-19.5	19.3	16.2-19.6	0.37
MMP-9	2.5	1.4-3.1	1.3	0.7-2.5	0.05	1.7	1.1-3.2	1.4	0.5-2.9	0.31
MMP-10	17.9	16.5-20.2	16.3	14.4-17.7	0.01	11.3	7.4-14.2	13.5	11.2-14.9	0.06
MMP-11	14.4	13.2-16.3	13.7	12.5-14.4	0.10	18.5	15.4-19.5	19.0	17.9-19.9	0.21
MMP-12	19.8	18.8-21.1	18.9	18.0-19.7	0.17	19.3	18.8-19.9	19.0	18.2-19.9	0.50
TIMP-1	2.7	2.1-3.1	2.8	2.0-3.5	0.47	2.2	1.6-2.9	2.6	2.2-2.9	0.09
TIMP-2	2.6	2.3-2.9	2.5	2.1-3.3	0.87	4.0	3.5-4.4	3.7	2.9-4.7	0.45

Table 12 Transcript abundance as measured by delta CT values for MMP genes after 24 hours of *in vitro* culture in the presence or absence of heat killed *M. Tuberculosis.*

Median 24 hour delta CT values

	unstimulated					stimulated				
mRNA	IRIS	IQR	non-IRIS	IQR	**p-value**	IRIS	IQR	non-IRIS	IQR	**p-value**
MMP-1	15.6	10.8-16.9	15.4	13.4-18.1	0.61	5.6	2.0-8.5	9.8	7.0-11.6	0.002
MMP-2	7.2	5.5-8.3	6.3	5.1-8.0	0.40	9.4	8.4-10.5	9.4	6.9-10.5	0.66
MMP-3	19.0	14.2-20.1	18.7	18.0- 19.4	0.66	20.1	10.2-20.8	17.2	16.9-19.6	0.35
MMP-7	9.4	8.3-10.4	9.3	8.0-10.0	0.80	6.8	4.8-8.6	8.7	7.34-9.66	0.01
MMP-8	19.0	13.4-20.3	18.9	17.6-20.6	0.74	19.5	15.0-20.2	17.2	14.1-19.8	0.56
MMP-9	2.8	1.5-3.3	2.3	1.4-3.9	0.82	0.3	-0.2 -1.4	0.7	0.1-1.9	0.25
MMP-10	18.3	12.4-19.0	18.1	16.1-18.8	0.95	10.5	7.1-11.6	13.4	12.1-16.3	<0.001
MMP-11	18.5	14.2-19.6	15.5	14.5-18.8	0.35	16.0	14.6-19.6	19.3	14.8-20.2	0.45
MMP-12	20.2	19.0-21.5	18.7	17.5-19.9	0.01	13.8	10.3-19.9	14.9	11.1-19.6	0.56
TIMP-1	3.0	2.4-3.5	3.5	3.1-4.2	0.02	2.6	1.6-3.0	3.4	2.4-4.4	0.02
TIMP-2	2.3	1.7-4.4	2.1	1.6-2.8	0.49	3.7	3.2-3.9	3.7	2.8-4.0	0.88

4.2.3 **Fold induction analysis of MMP genes**

The delta delta (Δ ΔCT) method used was used for analysis of RT-PCR data. Beta actin was used as the housekeeping gene through out these experiments. Delta CT was obtained by calculating the difference of the Ct of Beta Actin from the CT of the MMP gene of interest. Delta delta CT was then calculated by subtracting the delta CT of the unstimulated sample from the delta CT of the stimulated sample to obtain a delta delta (Δ Δ) Ct. This value was then substituted into the mathematical equation $2^{-\Delta\Delta CT}$ to obtain the fold induction/repression before log 10 transformation in order to normalise the data (Figure 15). Figure 15 shows a summary of the log fold induction of cytokine genes by heat killed *M. tuberculosis* for TB-IRIS and non-TB-IRIS control participants. At 6 hours, induction was significantly higher in TB-IRIS than in controls for MMP-3, MMP-7 and MMP-10 ($p \leq 0.05$). At 24 hours significant differences existed between TB-IRIS and non-IRIS for MMP-1, MMP-7, MMP-10, and MMP-12. Thus, MMP-7 and MMP-10 were consistently higher at both 6 and 24 hours in TB-IRIS patients compared to controls. By contrast as with the transcript levels, the genes for MMP-2, MMP-8, TIMP-2 and MMP-11 were down regulated by *M. tuberculosis* stimulation at both 6 and 24 hours in both the TB-IRIS and non-IRIS control groups. MMP-12 was undetectable in 6-hour cultures but was marginally induced in TB-IRIS at 24 hours.

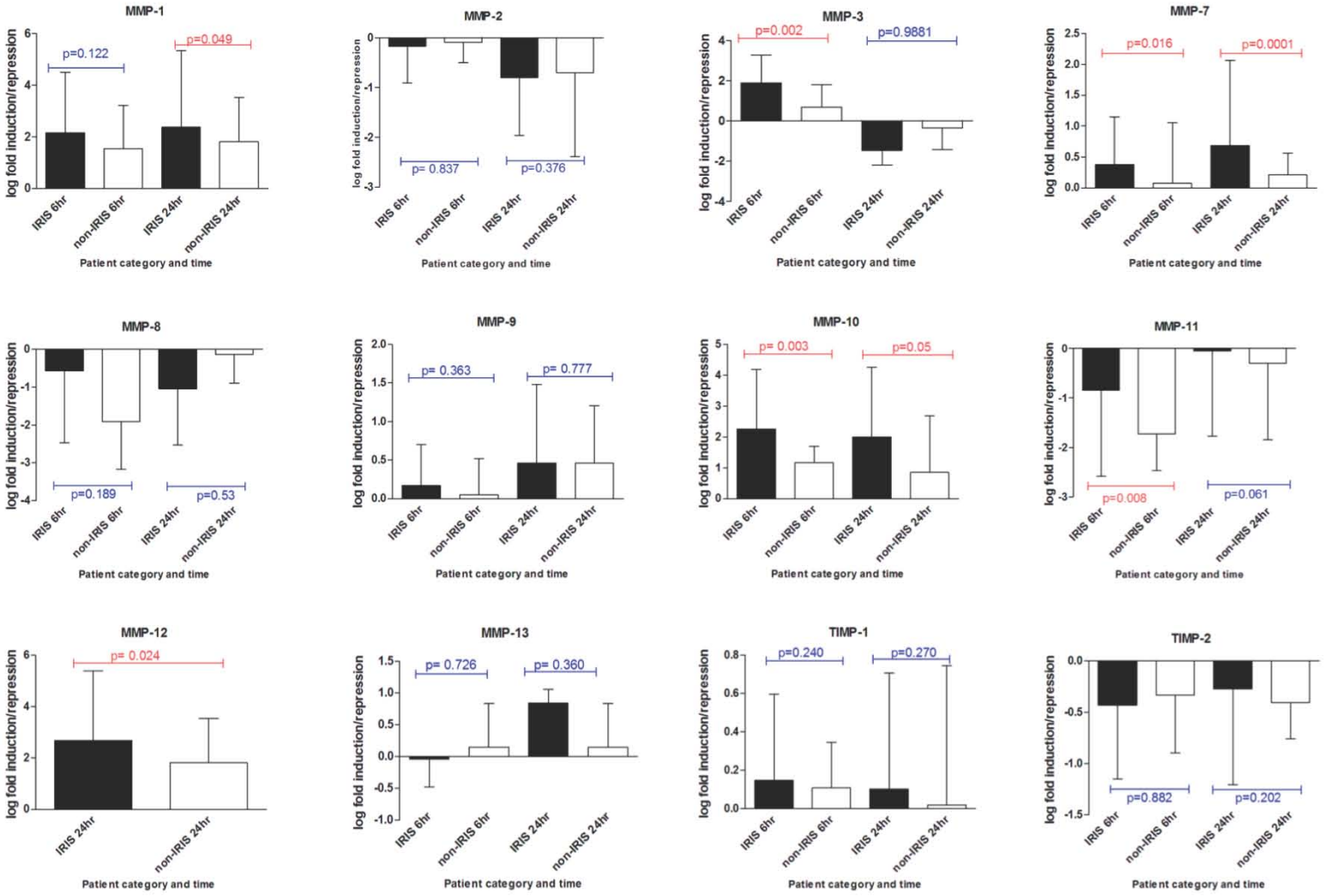


Figure 15 Summary of fold induction/ repression of the different MMP genes

4.2.4 **MMP protein secretion *(in vitro)* into tissue culture supernatant**

MMP levels were assayed in the tissue culture supernatants arising from 20 IRIS and 20 non-IRIS patients (based on sample availability). MMP protein secretion into corresponding tissue culture supernatants from the 24-hour *M. tuberculosis* stimulated PBMC was assessed mainly by luminex with the exception of MMP-10 and TIMP-1/2 concentrations which were measured by ELISA. Consistent with the gene expression data, secretion of MMP-1, -3, -7, and -10 protein was significantly higher in the tissue culture supernatants from TB-IRIS when compared to the controls ($p \leq 0.05$) (Figure 16). While TIMP-1 was expressed in very small amounts in both groups, TIMP-2 was completely undetectable in these cultures. MMP-2 was down regulated in both TB-IRIS and controls with no significant difference between the two groups. Although large amounts of MMP-9 were detected in these cultures, this did not differ significantly between TB-IRIS and non-IRIS controls.

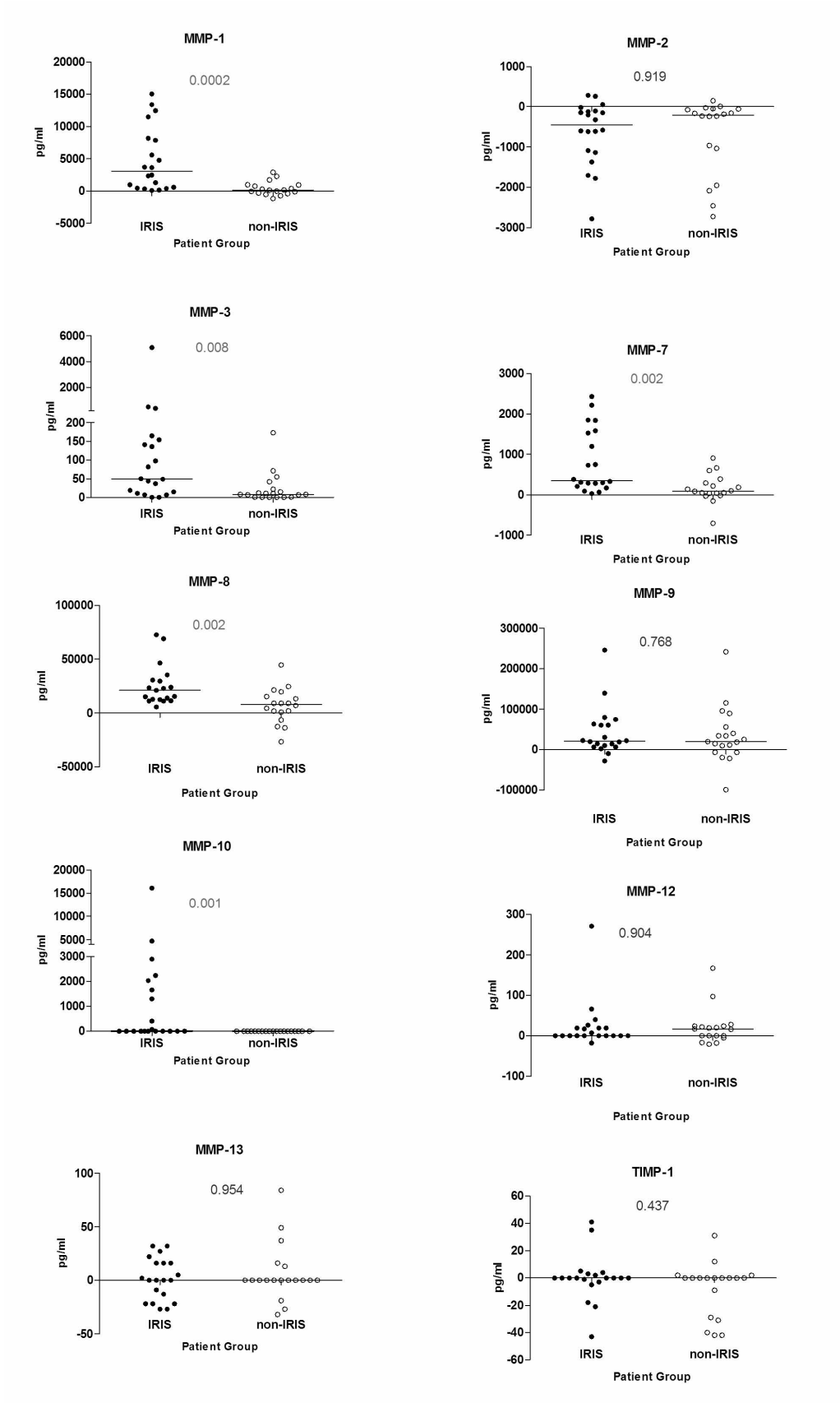


Figure 16 MMP protein secreted into tissue culture supernatants

4.2.5 **Correlation of MMP transcript vs. Protein secretion**

To determine the correlation between transcript and protein secretion, MMP transcript levels (Δ Ct values) for 6 and 24 hours were correlated (Spearman correlation) with the corresponding 24 hour tissue culture supernatant concentrations (pg/ml) for both TB-IRIS and non-IRIS controls. Figure 17 and 18 show a summary of these correlations at 6 and 24 hours respectively. In general there was an inverse correlation with higher transcript abundance (represented by smaller ΔCt values) corresponding to higher concentrations of protein secreted. There was a good correlation between MMP transcript and protein for MMP-2, MMP-7 and MMP-9 (Figure 17 and Figure 18) at both 6 and 24 hours. MMP-1 transcript correlated with protein secretion in the unstimulated 6-hour and the 24 hour stimulated samples only. For MMP-3 the correlation with secreted protein was significant with the 6 hour cultures on stimulation but not with the others. MMP-10 was not detected in non-IRIS patients and was detected only in very few IRIS patients. Corrections were not performed for multiple comparisons in this figure.

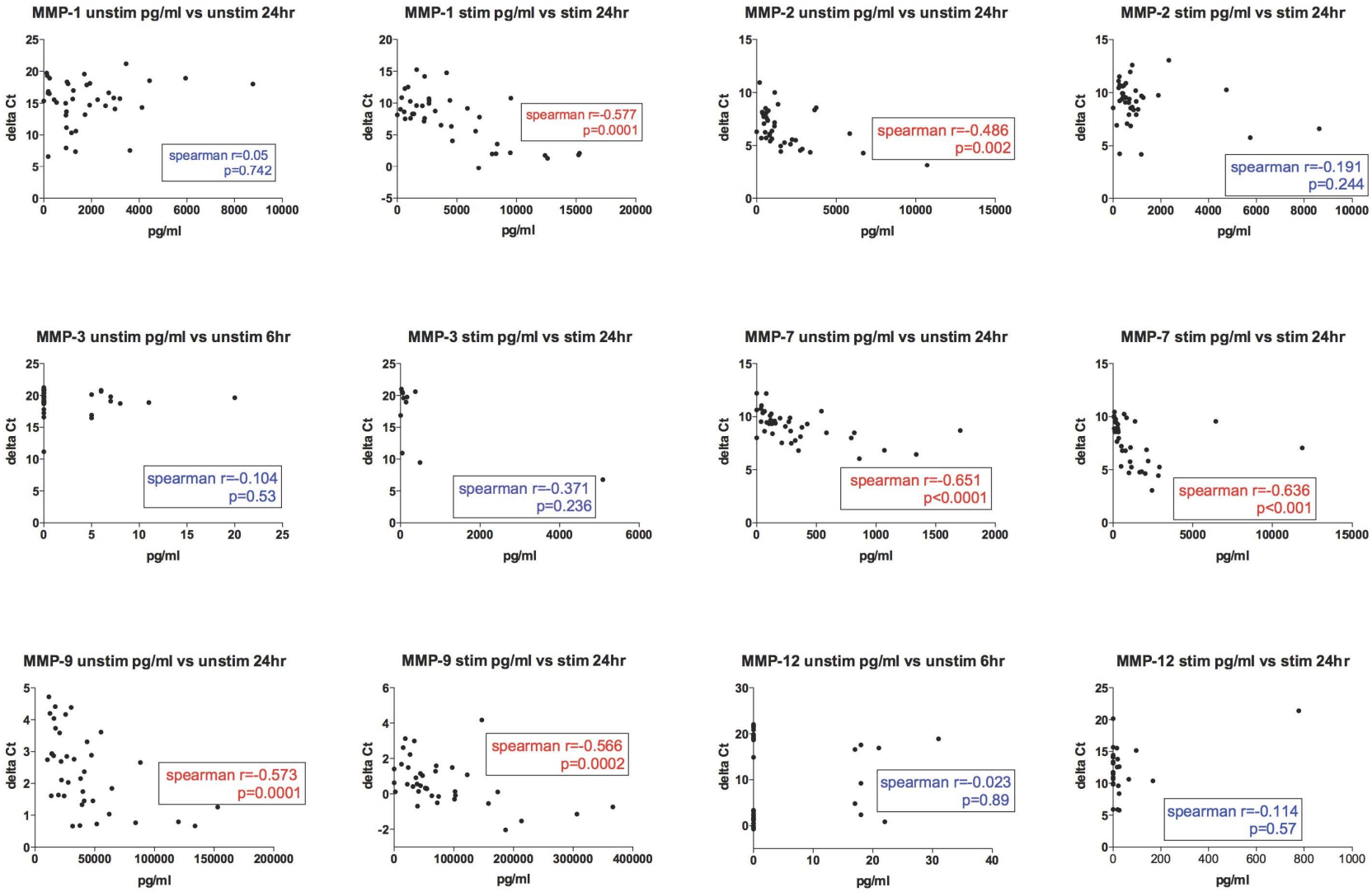
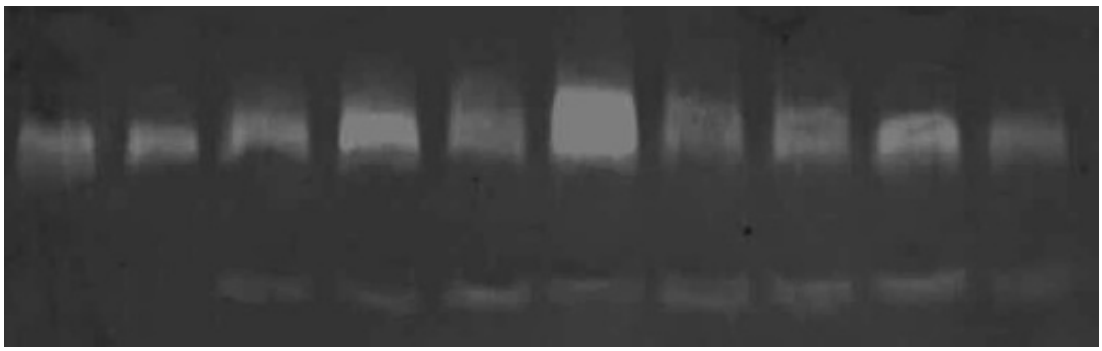


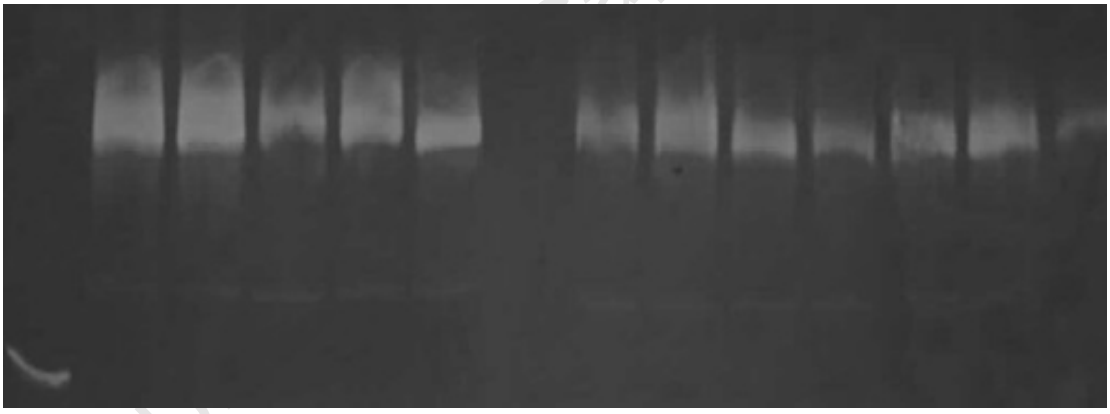
Figure 17 Correlation of MMP transcript with secreted protein in 24 hour tissue culture supernatants

4.2.6 **Enzymatic activity of MMP-9 as determined by gelatin zymography**



	MMP-9	MMP-9	NI 1 U	NI 1 S	I 1 U	I 1 S	NI 2 U	NI 2 S	I 2 U	I 2 S
MMP-9 pg/ml	-	-	38209	72369	26662	101281	43655	36660	48451	38597
Area under curve	-	-	450.95	658.17	472.62	892.77	892.77	361.59	492.39	267.68

Figure 19a



	MMP-2	NI 3U	NI 3S	I 3 U	I 3 S	NI 4 U	NI 4 S	I4 U	I4 S	NI 5 U	NI 5 S	I 5 U	I 5 S	MMP-9
MMP-9 pg/ml	-	N.D	N.D	40053	70651	51698	63116	55254	69541	21802	15170	15643	22183	-
Area	-	1714.5	1681	1003.5	1441.7	1029.3	N.D	629.79	918.9	616.9	480.72	657.28	977.33	-

Figure 19b

Figure 18 Representative Gelatin zymograms

Figure 19a and 19b show two representative gelatin zymograms performed on 24 hour tissue culture supernatants. The intensity of the clear bands was determined by Scion Image analysis to give area under the curve. Corresponding luminex results in pg/ml these TB-IRIS

patients (I) and non-IRIS controls (NI), U= unstimulated sample, S= stimulated sample, ND= not detected. MMP-2 and MMP-9 on these figures represent positive controls. MMP protein retained potential for proteolytic activity despite there being no distinct differences between IRIS and non-IRIS in the area under the curve, hence in the level of MMP activity

MMP-9 protein levels measured by luminex tended to relate well to the area under the curve (measured by gelatin zymography) in the same sample although there were no significant differences noted between TB-IRIS and non-IRIS control samples. Luminex measures total protein in a sample (in this case MMP-9), while zymography measures enzymatically active MMP-9 by measuring the intensity of the clear lysed band on the zymogram.

4.2.7 **MMP levels in serum *(in vivo)***

To confirm whether the increased MMP expression and secretion detected in the stimulated PBMC was reflected *in vivo*, we analyzed circulating MMPs in corresponding serum samples of the TB-IRIS and control participants. MMP levels were assessed in 22 TB-IRIS and a 22-non-IRIS control by luminex while MMP-10 protein was measured by ELISA. MMP-7 was significantly higher in the serum of TB-IRIS participants compared to controls (p=0.001). Levels of TIMP-1 were also higher in the IRIS group (p=0.003). Of the other MMPs analysed including MMP-3, MMP-10 and TIMP-2, no differences were noted in the serum MMP levels between TB-IRIS participants and controls. Figure 20 shows a summary of these results. Corrections were not made for multiple comparisons in this figure.

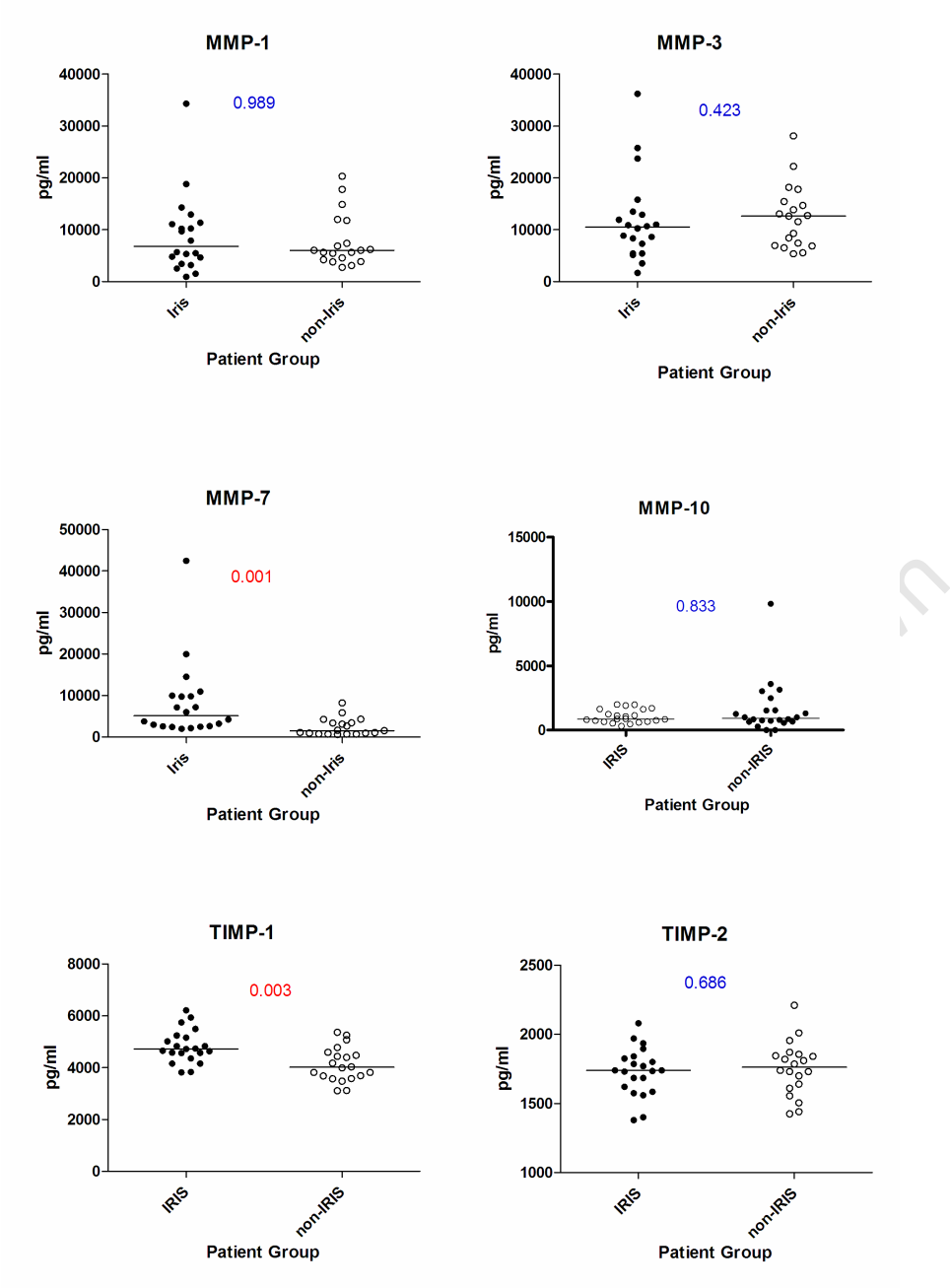


Figure 19 Summary of MMP protein levels in the serum samples of TB-IRIS and non-IRIS patients.

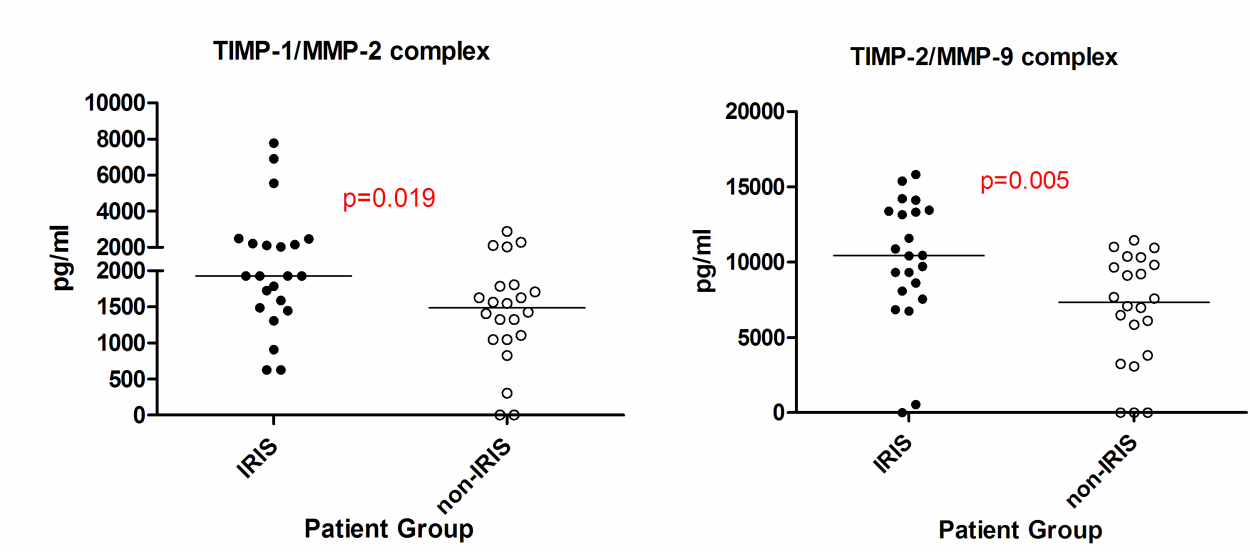


Figure 20 Serum (*in vivo)* levels of TIMP complexes

The concentrations of TIMP-2 complexed with MMP-9 (TIMP-2/MMP-9) and TIMP-1 complexed with MMP-2 (TIMP-1/MMP-2) circulating in serum samples were also assessed. The concentrations of the TIMP-1/MMP-2 and TIMP-2/MMP-9 complexes were observed to be higher in TB-IRIS than in non-IRIS patients. The concentrations of TIMP-1/MMP-2 and TIMP-2/MMP-9 complexes were significantly higher in the serum of TB-IRIS compared to non-IRIS patients (Figure 21).

4.2.8 **Effect of Corticosteroid therapy on gene expression and serum levels in TB-IRIS**

Corticosteroid therapy has been shown to improve clinical outcomes in patients presenting with paradoxical TB-IRIS (Meintjes, Wilkinson et al. 2010). To determine the effect of corticosteroid therapy on levels of MMP protein in the serum *(in vivo)* of TB-IRIS patients who were enrolled in the prednisone-placebo RCT and were treated with either a 4-week course of prednisone or placebo were compared. MMP-1, MMP-3, MMP-7 and MMP-10 gene expression was analysed by quantitative RT-PCR in *M. tuberculosis*-stimulated PBMC cultures from 16 TB-IRIS participants treated with prednisone therapy compared to 12 who were placebo-treated. Figure 22 and 23 show a summary of these analyses in the 6 and 24-hour cultures respectively.

Prednisone treatment did not significantly modulate MMP gene expression in the 6-hour cultures (Figure 22). However, in the 24-hour cultures, MMP-7 and MMP-10 were significantly suppressed in the prednisone-treated but not in the placebo-treated group (Figure 23). Prednisone therefore significantly suppressed MMP-7 gene expression over the treatment course ($p=0.006$). MMP-10 was also marginally suppressed ($p=0.045$). MMP-1 and MMP-3 genes were not modulated by either prednisone or placebo treatment over the treatment course.

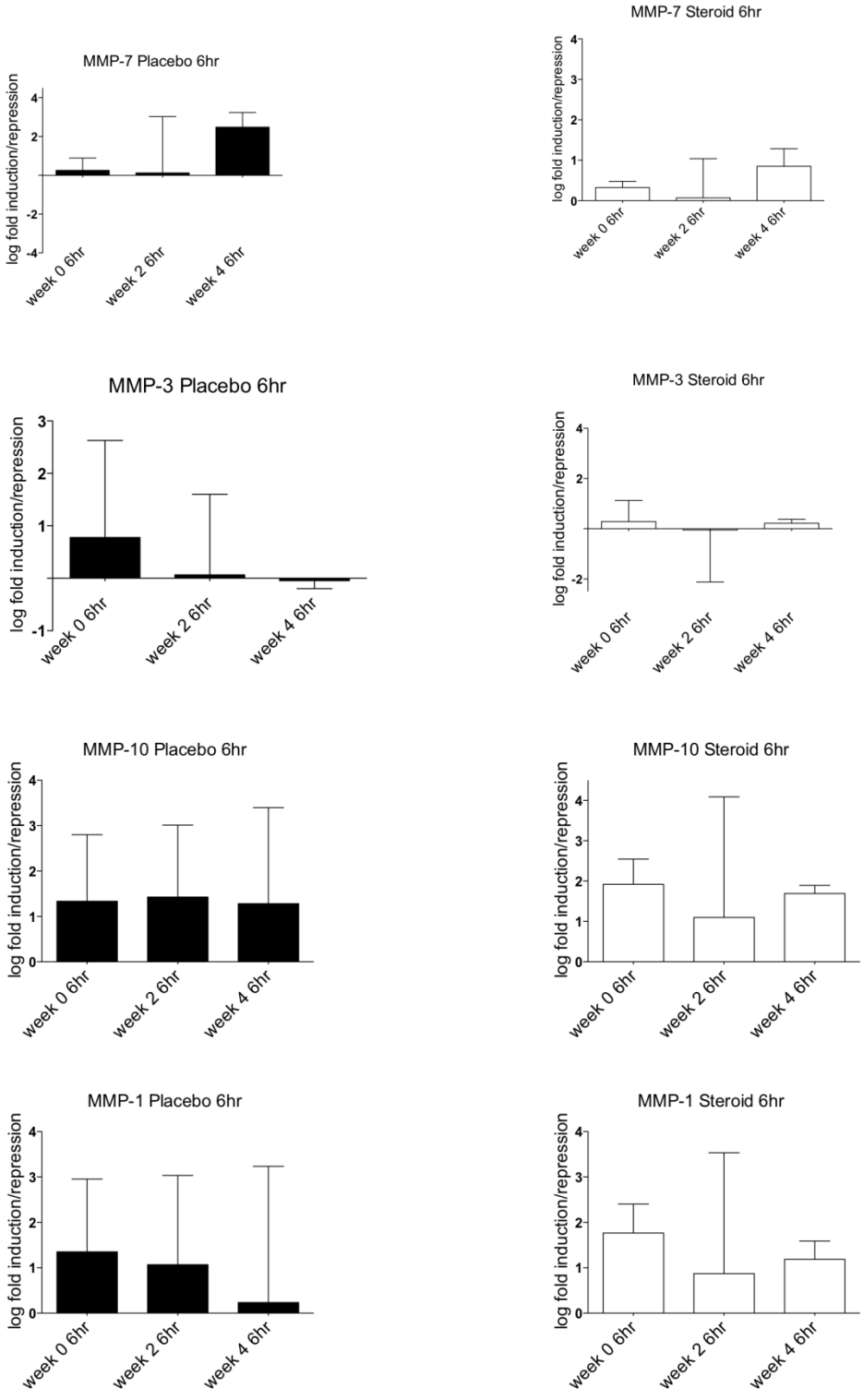


Figure 21 Effect of Prednisone on MMP gene induction/repression at 6 hours

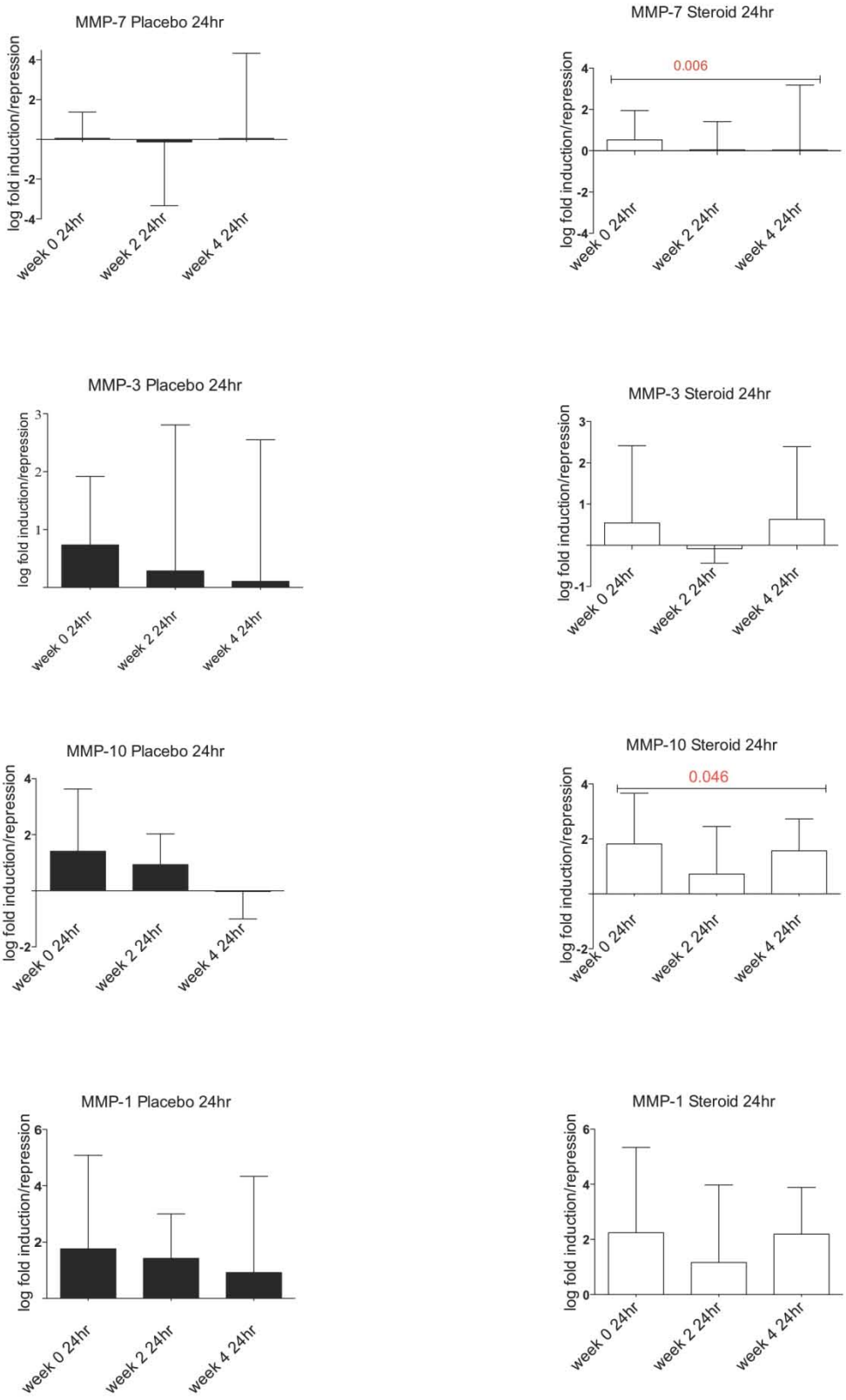


Figure 22 Effect of Prednisone on MMP gene induction/repression at 24 hours

4.2.9 **Effect of corticosteroid therapy on the levels of circulating MMPs**

Finally the levels of circulating MMP-7 in the corresponding serum samples of these RCT participants over 4 weeks *of treatm*ent were analysed. Prednisone tended to significantly suppress circulating MMP-7 levels (p=0.046) between week 0 and week 2 (Figure 24). In the placebo treated group, there was no difference in the MMP-7 levels over the treatment course. MMP-1 and MMP-3 levels did not appear to be modulated by prednisone or placebo in these patients.

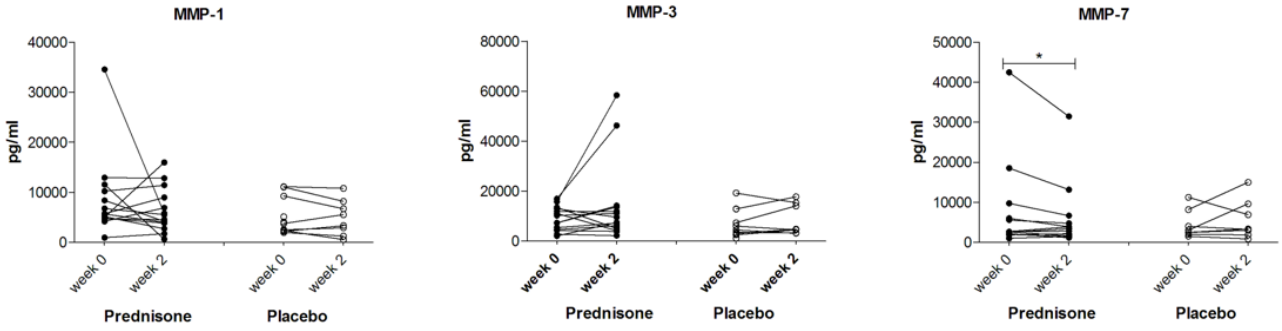


Figure 23 Analysis of the effect of prednisone treatment on MMP levels in TB-IRIS

4.3 Discussion

In summary, this chapter presents a comprehensive series of studies that was conducted to investigate the role of tissue degrading enzymes (MMPs) in participants who developed paradoxical TB-IRIS. The findings show that *M. tuberculosis* stimulation of PBMC increased the transcript levels for MMP-1, MMP-7, MMP-10, and TIMP-1 genes in paradoxical TB-IRIS participants in 24-hour cultures ($p \leq 0.02$). Fold induction was significantly higher in TB-IRIS than in controls for MMP-3 at 6 hours, while in the 24-hour cultures MMP-1 and MMP-12 were differentially induced in TB-IRIS. MMP-7 and MMP-10 were differentially induced in TB-IRIS at both 6 and 24 hours.

There was consistency between gene expression and supernatant results with higher MMP protein levels being observed in the tissue culture supernatants for MMP-1, -3, -7 and -10 genes which were higher in TB-IRIS. This was also supported by the results of the Spearman correlation analysis (correlation coefficients and the significant p values) for these MMPs, which indicates that control is mainly transcriptional. The up-regulation of MMP-1, -3, -7 and -10 by *M. tuberculosis* is consistent with previous reports in primary human macrophages, monocytes (Harris, Green et al. 2007; Elkington, Green et al. 2009) and PBMC. MMP-7 is expressed in macrophages within TB granulomas. Analysis of serum samples showed MMP-7 and TIMP-1 protein levels to be higher *in vivo* for TB-IRIS compared with controls. Prednisone has been shown to improve clinical outcomes in TB IRIS patients (Meintjes, Wilkinson et al. 2010) . In a subset of paradoxical TB-IRIS participants randomised to prednisone or placebo treatment, MMP-7 and MMP-10 genes were shown to be significantly down regulated in steroid but not in the placebo treated participants. Analysis of the concentrations of circulating MMP-7 showed a significant decrease over the first two

weeks of treatment in the prednisone but not in the placebo treated group. Taken together, these data implicate MMP-7 in the immunopathology of TB-IRIS.

The involvement of matrix metalloproteinases in lung biology and in the tissue degrading phenotype of pulmonary tuberculosis has been reported previously (Parks and Shapiro 2001; Parks, Wilson et al. 2004; Elkington and Friedland 2006). MMP-7 has been shown to be highly expressed in macrophages within TB granulomas (Elkington, Nuttall et al. 2005; Elkington, Shiomi et al. 2011). However, this is the first study to investigate the involvement of these tissue-degrading enzymes in paradoxical TB-IRIS. The clinical features and symptoms observed in patients presenting with TB-IRIS suggest an association of this condition with tissue damage. I have shown that stimulation of PBMC with *M. tuberculosis* upregulates several of the MMP genes in TB-IRIS patients. These findings corroborate related findings on analysis of gene expression and secretion following direct *M. tuberculosis* infection of human microglia in a cellular model of CNS TB (Green, Tran et al. 2009). My findings suggest that MMPs are involved in TB-IRIS pathogenesis and that infection with MTB promotes a tissue damaging phenotype, which is evident in TB-IRIS (Meintjes, Lawn et al. 2008).

While MMP-9 was highly expressed in the patients analysed in these studies, there was no difference in the levels of this MMP in both the mRNA and secreted protein between TB-IRIS and non-IRIS. Analysis of MMP-9 enzymatic activity by zymography showed the presence of active MMP-9 in both patient groups and this tended to relate well to the MMP-9 levels as measured by luminex. MMP-9 has been implicated in tuberculosis immunopathology (Friedland, Shaw et al. 2002; Sheen, O'Kane et al. 2009). More recently, MMP-9 has been implicated in the regulation of monocyte recruitment in granuloma formation and bacterial growth in the *M. marinum* model of infection in zebra fish,

suggesting a potential host-targeting tuberculosis therapeutic approach targeting the disruption of MMP-9 function (Volkman, Pozos et al. 2010). I did not demonstrate divergent MMP-9 gene expression or secretion between the IRIS and non-IRIS patients. Thus, while MMP-9 may be associated with the pathogenesis of tuberculosis, it does not appear to be specifically associated with the immunopathology that is evident in TB-IRIS.

MMP-7 correlated closely with IRIS and was suppressed by prednisone treatment. MMP-7 may act as a regulator of the immune response as opposed to directly cleaving fibrils of the extracellular matrix (Parks, Wilson et al. 2004). MMP-7 is a multifunctional MMP, which is required for activation of defensins (Wilson, Ouellette et al. 1999). For example, MMP-7 regulates neutrophil eggression into the lung by cleaving syndecan-1 to then generate a chemotactic gradient (Li, Park et al. 2002) and can also release pro-TNF alpha from the cell-surface (Haro, Crawford et al. 2000). In other studies, MMP-7 has been shown to regulate the lung localisation of dendritic cells to limit inflammation and inhibit fibrosis (Manicone, Huizar et al. 2009). MMP-7 activity has also been previously implicated in the pathogenesis of aberrant lung remodelling in pulmonary fibrosis (Zuo, Kaminski et al. 2002; Rosas, Richards et al. 2008).

Tissue inhibitors of matrix metalloproteinases (TIMPs) are known to down-regulate MMP-activity by binding to latent and active forms of MMPs. While TIMPs are constitutively expressed in many tissue fluids, including CSF (Rosenberg 2002), the peripheral measurement of these inhibitors may not always reflect tissue effects (Price, Farrar et al. 2001) . This may explain the finding in this study that the TIMP genes were generally not regulated by *M. tuberculosis* at both the mRNA and tissue culture supernatants levels, but were then found elevated in the serum of TB-IRIS participants. The presence of TIMP-

1/MMP-2 and TIMP-2/MMP-9 complexes in significantly higher levels in the serum of IRIS patients than detected *in vitro* may suggest that active forms of these MMPs are present *in vivo* as complexes with these tissue inhibitors. Endogenous inhibitors including α -macroglobulin and other TIMPs exert their effect in vivo by trapping active MMPs, with MMP-1 known to be rapidly inhibited by α2-macroglobulin which is particularly abundant in serum (Visse and Nagase 2003). Thus, the regulatory mechanisms associated with controlling the potentially destructive nature of MMPs by binding active MMPs with their endogenous inhibitors may complicate the study and quantification of MMPs in biological samples. While I showed high levels of various MMP proteins *in vitro*, but not *in vivo*, I detected the presence of MMP-2/TIMP-1 and MMP-9/TIMP-2 complexes. These findings suggest a possible inhibition of active MMPs by TIMPs in the serum samples, and hence their availability and subsequent detection as complexes.

In summary, the findings documented in this chapter show that stimulation with *M. tuberculosis* differentially up-regulates multiple MMPs in TB-IRIS patients. MMP-7 was consistently expressed in increased levels in participants with TB-IRIS and was suppressed by prednisone. However, since steroids have multiple deleterious immunological effects and may be harmful in the context of drug-resistant TB, more specific immunomodulatory approaches are needed in TB-IRIS. Dysregulated MMP activity may represent a therapeutic target to reduce immunopathology without causing steroid-related immunocompromise. These findings indicate an involvement of MMPs, particularly MMP-7 and MMP-10 in the immunopathology of paradoxical TB-IRIS. Modulation of dysregulated MMP-7 activity during TB-IRIS may represent a novel therapeutic approach to alleviate this condition.

5 CHAPTER 5: MMPS AS MODULATORS OF INFLAMMATION IN THE IMMUNOPATHOLOGY OF HIV-TB IRIS

5.1 Introduction

The role of MMPs was originally believed to be restricted to the degradation of the extracellular matrix (ECM) to permit normal remodelling and contribute to pathological tissue destruction (McCawley and Matrisian 2001). However, recent evidence, particularly from MMP-deficient mice shows the involvement of MMPs in modifying other non-matrix substrates such as chemokines, growth factors, receptors and or cytokines (Van Lint and Libert 2007). Thus MMPs can influence the progression of various physiological and inflammatory processes. Inflammation, though essential for host defence and tissue repair, can contribute to ongoing tissue injury (Manicone and McGuire 2008). Inflammatory and tissue destroying conditions such cancer, rheumatoid arthritis and periodontitis have been shown to be associated with dysregulated and often increased levels and activities of several MMPs (Parks, Wilson et al. 2004).

In general, MMPs contribute to various inflammatory processes by modulating inflammatory mediators, establishing chemokine gradients in inflamed tissues (McCawley and Matrisian 2001; Manicone and McGuire 2008). Thus MMPs can either protect or contribute to immunopathology in inflammatory processes by facilitating leucocyte recruitment, chemokine activation, generation of chemokine gradients and cytokine processing (Woessner and Nagase 1999; Visse and Nagase 2003). Leucocyte migration is a hallmark of inflammation which is primarily triggered and regulated by chemoattractant chemokines (RodrÌguez, Morrison et al. 2010). Precise MMP processing can inactivate, enhance or antagonise the chemotactic properties of chemokines, thus influencing the final outcome of

inflammation. MMP-mediated proteolysis of chemokines is one way by which MMPs have been shown to influence leucocyte trafficking. The proteolysis of chemokines by MMPs may inactivate the chemokines, generate antagonistic derivatives or produce more potent chemoattractant products than the full-length molecules but may also lead to the inactivation of other chemokines (Van den Steen, Proost et al. 2000; Van Lint and Libert 2007). For example, cleavage of IL-8 by MMP-9 (gelatinase B) has been show to produce a truncated product of IL-8 which has ten-fold greater activity (Opdenakker, Van den Steen et al. 2001) than the original product. MMPs may also indirectly control influx of inflammatory cells by cleaving proteins in the pericellular environment that binds chemokines. Some studies have shown that MMP-7 is required to generate a chemokine gradient, while MMP-8 and MMP-9 have also been shown to promote neutrophil migration (Li, Park et al. 2002; Corry, Kiss et al. 2004; Van Lint, Wielockx et al. 2005).

The processing of cytokines may lead to the formation of more potent intermediates of the cytokines with altered bioavailability and activity by direct proteolytic processing or sometimes by shedding their receptors (Van Lint and Libert 2007). TNF, IL-1beta, IL-2 and TGF-Beta are some of the inflammatory cytokines whose activity has been shown to be amenable to MMP proteolysis. Several MMPs including MMP-1, -2, -3, -9, -7 and -12 have been shown to have the ability to convert pro-TNF to the potent pro-inflammatory cytokine, TNF in activated macrophages (Mohan, Seaton et al. 2002). Similarly, MMP-2, -3, and -9 has been shown to convert the IL-1β precursor to the active form of the enzyme (Schonbeck, Mach et al. 1998). Interestingly, MMP-3, MMP-1, -2 and -9 have been shown to degrade the active IL-1β, suggesting opposing roles of MMPs in either repressing or promoting IL-1β effects (Ito, Mukaiyama et al. 1996). The role of MMPs in modulating inflammatory responses is complex and requires further investigation. Whether MMPs can influence the

outcome of inflammation and how they exert their function is a question that remains incompletely understood. Understanding this mechanism will help in developing effective drug targets to modulate MMP activity in inflammatory conditions (Van Lint and Libert 2007).

Tuberculosis-associated immune reconstitution inflammatory syndrome has been reported to be associated with exacerbated inflammation (Meintjes, Wilkinson et al. 2008). In general, the results reported in chapter 3 and 4 of this thesis showed distinctly higher levels for various cytokines and MMPs at different levels of analysis. I hypothesized that there may be correlations between the high levels of cytokines and MMPs in these patients. To synthesize these results and assess any relationship between high levels of cytokines and MMPs in these patients, the concentrations (those that were highly expressed in the 24 hour tissue culture supernatants) of different pro- and anti-inflammatory cytokines and chemokines were correlated with the concentrations of MMPs. This analysis is presented in this chapter. Table 13 below shows a summary of some of the functions of the established functions of MMPs in modulating inflammation.

Table 13 Summary of MMP functions in modulating inflammation

(Adapted from Chakraboti, 2003, Woeesner and Nagase 2000, McCawley, 2001

MMP	Non-traditional substrate	Resultant effect	Extracellular Matrix substrates	Activated by	Activator of
MMP-7/matrilysin	pro-α-defensin decorin pro-TNFα pro-MMP-2,-7, -9 MMP-9/TIMP-1 complex	α-Defensin Bioavailable TGF-β Bioavailable TFNα MMP-2, MMP-7, MMP-9	proteoglycans, laminin, fibronectin, gelatin, fibrin/fibrinogen, collagens,casein	MMP-2, MMP-3, MMP-10	MMP-2
MMP-1/collagenase-1	α1-antichymotrypsin a1-proteinase inhibitor Pro-MMP-1, -2 pro-TNFα MMP-2, MMP-9	Inactive serpin Inactive serpin MMP-1, 2 Bioavailable TNFα	collagens, gelatin, aggrecan, proteoglycan	MMP-3, MMP-10	MMP-2
MMP-8/collagenase 2	α1−proteinase inhibitor Pro-MMP-8	Inactive serpin MMP-8	collagens, gelatin, aggrecan	MMP-3, MMP-10, plasmin	ND
MMP-13/collagenase 3	α1-antichymotrypsin Pro-MMP-9, -13 plasminogen activator inhibitor-2	inactive serpin MMP-9, MMP-13	collagens, gelatin,fibronectin	MMP-2, MMP-9, -10, -14, -15, plasmin	MMP-2, MMP-9
MMP-3/stromelysin	α1−proteinase inhibitor Pro-IL-1β, Pro-TNFα Plasminogen α1-antichymotrypsin Pro-MMP−1, −3, −7, −8, −9, −13	Inactive serpin IL-1β, TNFα	collagens, gelatin, aggrecan, proteoglycan	plasmin, trypase	MMP-1, MMP-7, MMP-8, MMP-9, MMP-13
MMP-10/stromelysin 2	Pro-MMP-1, -8, -10	MMP-1, -8, -10	elastase, cathepsin G	MMP-1, -7, -8	MMP-1, MMP-7, MMP-8, MMP-9, MMP-13
MMP-11/stromelysin 3	α1-proteinase inhibitor, casein	Inactive serpin	laminin, fibronectin, agrrecan	Furin	ND
MMP-2/gelatinase A	decorin, pro-TGFβ2, Pro-IL-1b, MCP-3, Pro-TNF, Pro-MMP-1, -2, -13	bioavalable TGFB, inactive chemoattractant (MCP-3), TNF, MMP-1, -2, 13, active IL-1β	collagens gelatin, elastin,fibronectin, proteoglycan	MMP-1, -7, -13	MMP-9, MMP-13
MMP-9/gelatinase B	α1-proteinase inhibitor, Pro-TGFβ2 Pro-IL-1β, Pro-TNF, plasminogen	α1−proteinase inhibitor, IL-1β, plasminogen Bioavailable IL-1b, TNF, angiostanin	collagens,gelatin, elastin,fibronectin	MMP-2, MMP-3, MMP-13	ND

5.2 Results

To assess the immunomodulatory role of MMPs have in inflammation in TB-IRIS, correlations were determined between MMPs and various pro-and anti-inflammatory cytokines that were expressed in appreciable concentrations in the 24-hour tissue supernatants. The concentrations of 10 cytokines (IL-1β, IL-2, TNF, IL-6, IFN-gamma, IL-10, IL-12, IL-13, IL-10, GM-CSF) and 4 chemokines (IL-8, MIP-1α MIP-1β and RANTES) were correlated with 5 MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-10) and TIMP-1. For these analyses, all the concentrations were background subtracted before analysis by subtracting the concentration of the unstimulated sample from the stimulated sample. Thus, all analysis was done on the background-subtracted concentrations, hence explaining the negative concentrations for some of the samples (i.e in cases where the unstimulated value was higher than stimulated value). Spearman correlations were performed and the significant Spearman correlation coefficients (r) and the corresponding p-values are shown on the graphs.

5.2.1 Correlation of MMP with cytokine concentrations

Except for MMP-10 (where the correlation was on IRIS patients data since MMP-10 was only detected in tissue culture supernatants of some IRIS patients), the correlations shown in Figure 25 were on pooled data of IRIS and non-IRIS patients. Figure 25 shows a summary of the correlation of MMP-1 with the different pro and anti-inflammatory cytokines. There was a significant correlation between MMP-1 and IL-1β, TNF, IL-6, and IL-13 ($p< 0.013$) but not with IFN-gamma. MMP-2 (gelatinase A) was positively and significantly correlated (Figure 25) with IL-1β, TNF, IL-6, IL-10 and GM-CSF but not significantly correlated with IFN-

gamma. Although large amounts of (MMP-9) gelatinase B were detected in these cultures there were no significant correlations detected between MMP-9 and the various cytokines analysed. When the concentrations of MMP-3 were correlated to cytokine concentrations, positive and significant correlations were observed between MMP-3 and IL-1β, TNF, IL-6 and IL-10 but not IFN-gamma (Figure 26 and Figure 27). MMP-7 and MMP-10 correlated positively with IL-1β, GM-CSF, IL-13, TNF, IL-6 and IL-10 and this was highly significant (Figure 28 and Figure 29). Although large amounts of the neutrophil collagenase (MMP-8) were detected in these cultures, this did not show distinct correlations with any of the pro- or anti-inflammatory cytokines. While appreciable higher concentrations of TIMP-1 were observed in these cultures, there were no significant correlations of TIMP-1 with any of the cytokines that were analysed.

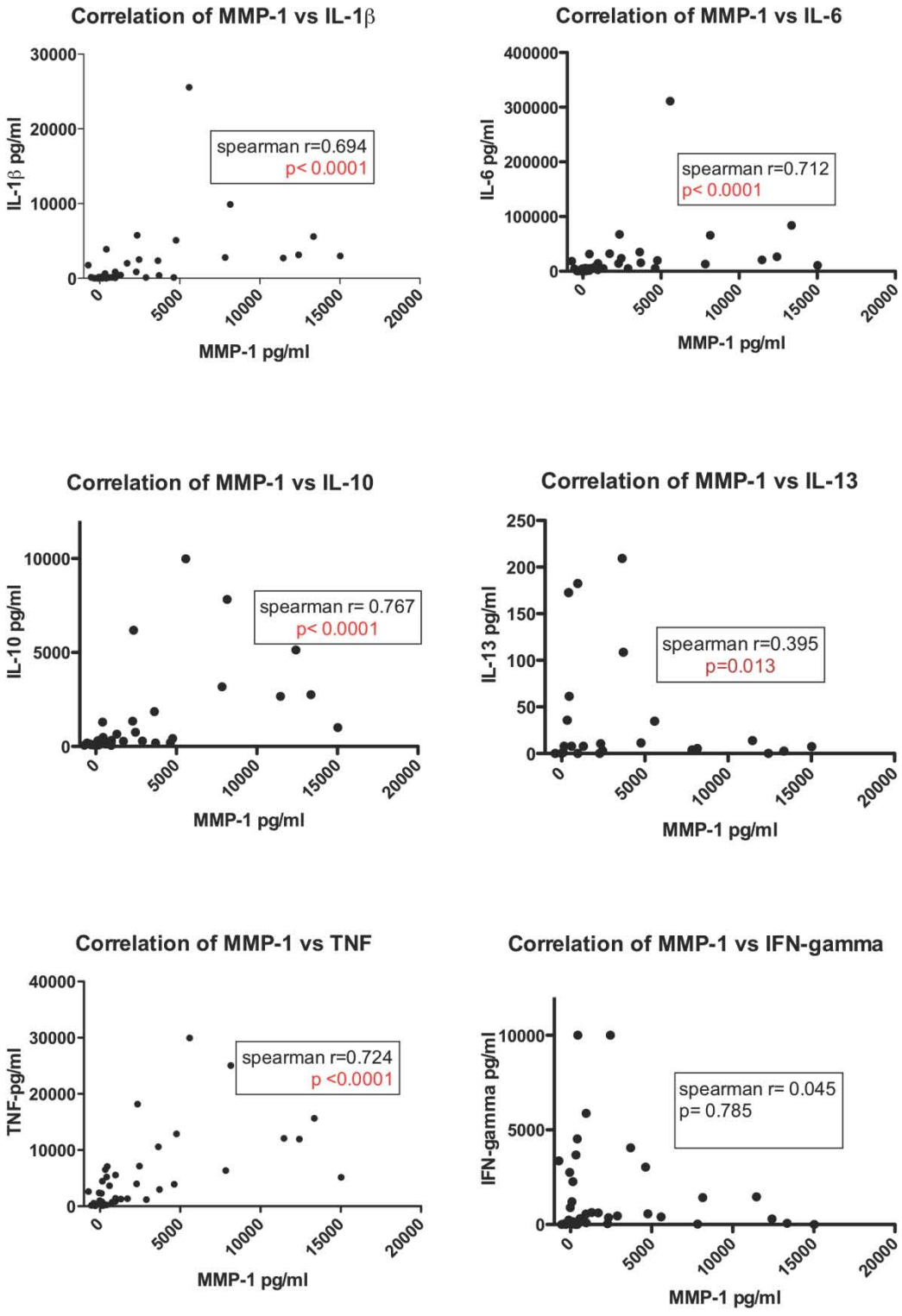


Figure 24 Correlation of MMP-1 vs. pro- and anti-inflammatory cytokines

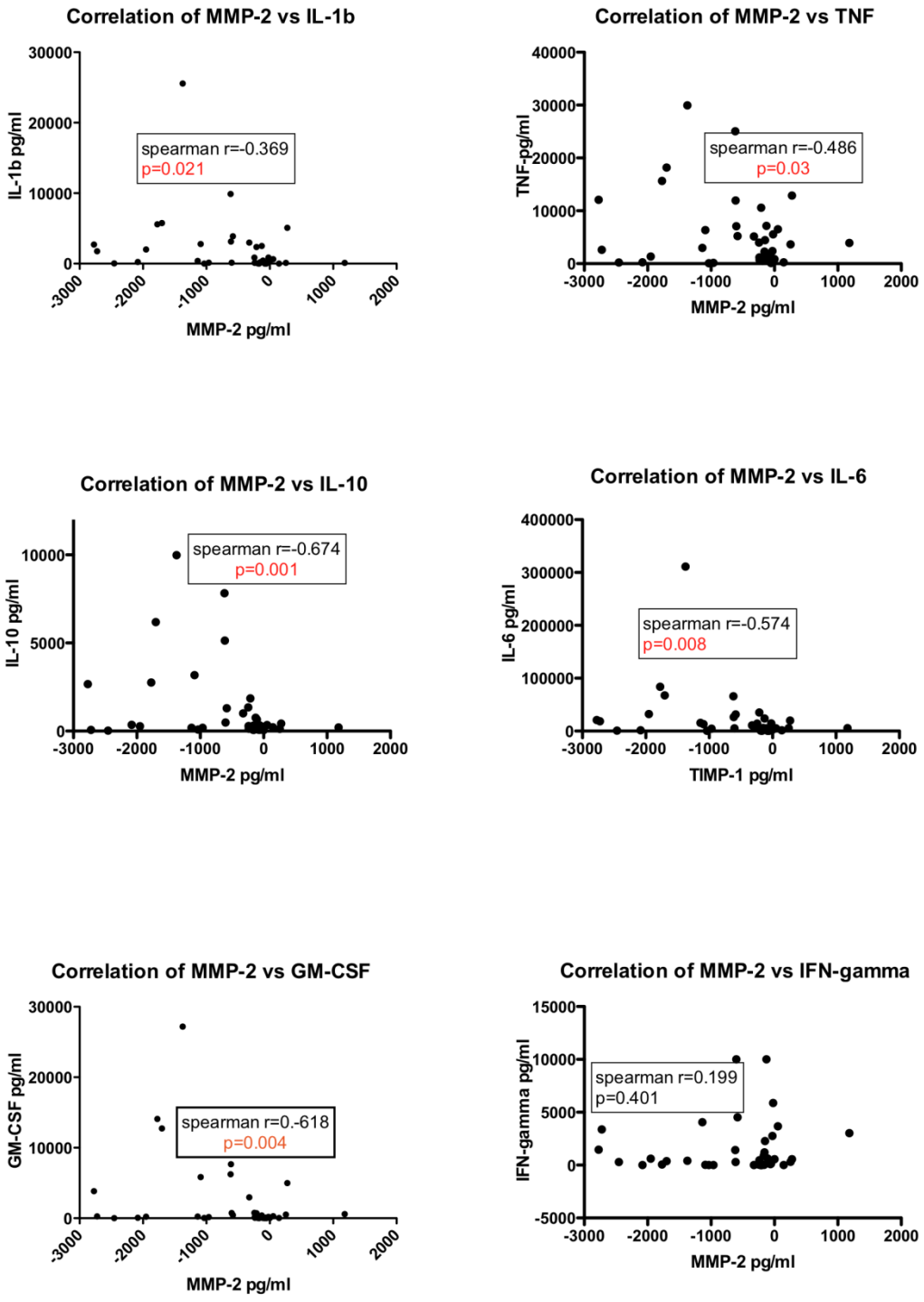


Figure 25 Correlation of MMP-2 vs. pro-and anti-inflammatory cytokines

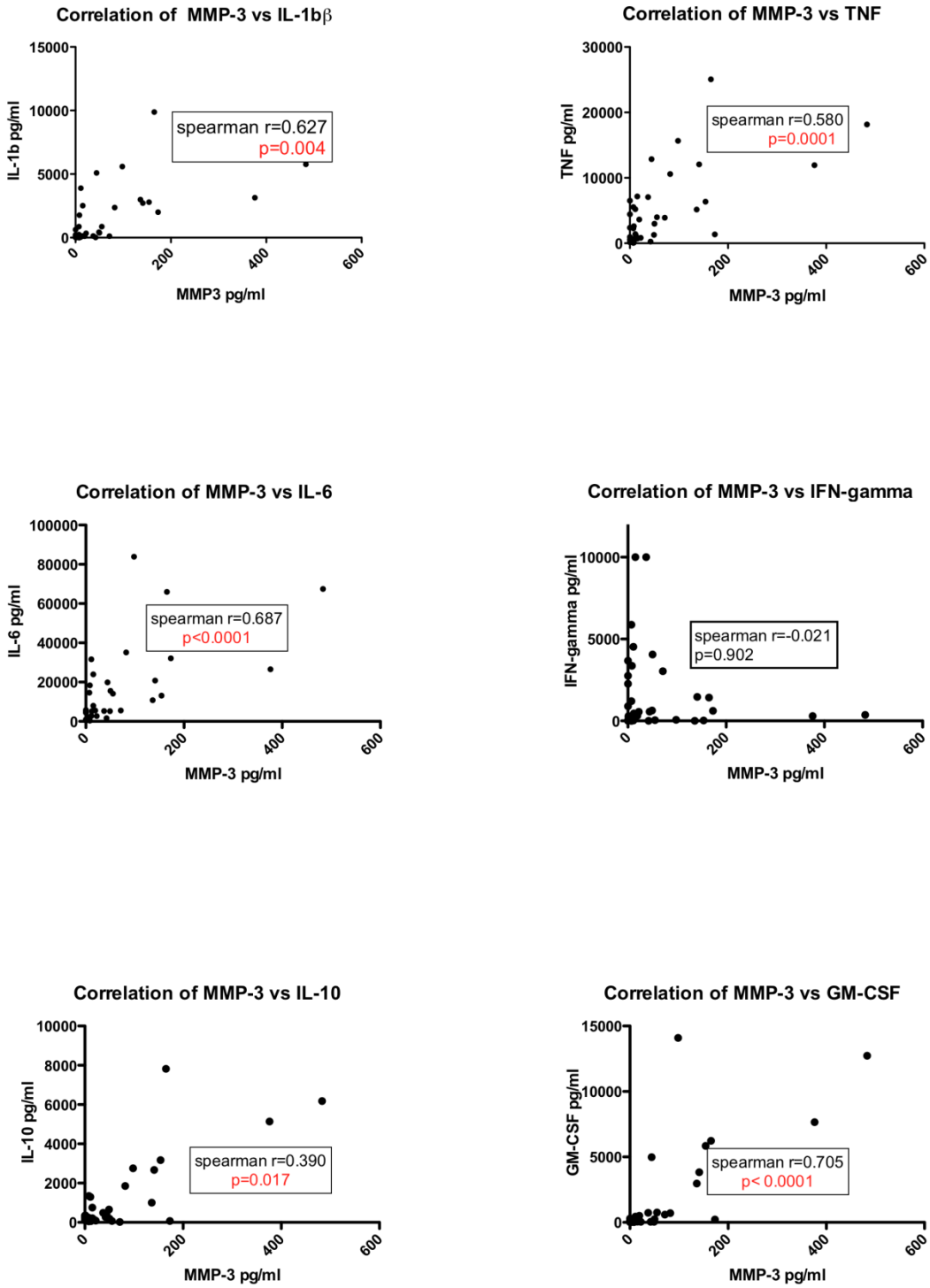


Figure 26 Correlation of MMP-3 vs. pro-and anti-inflammatory cytokines

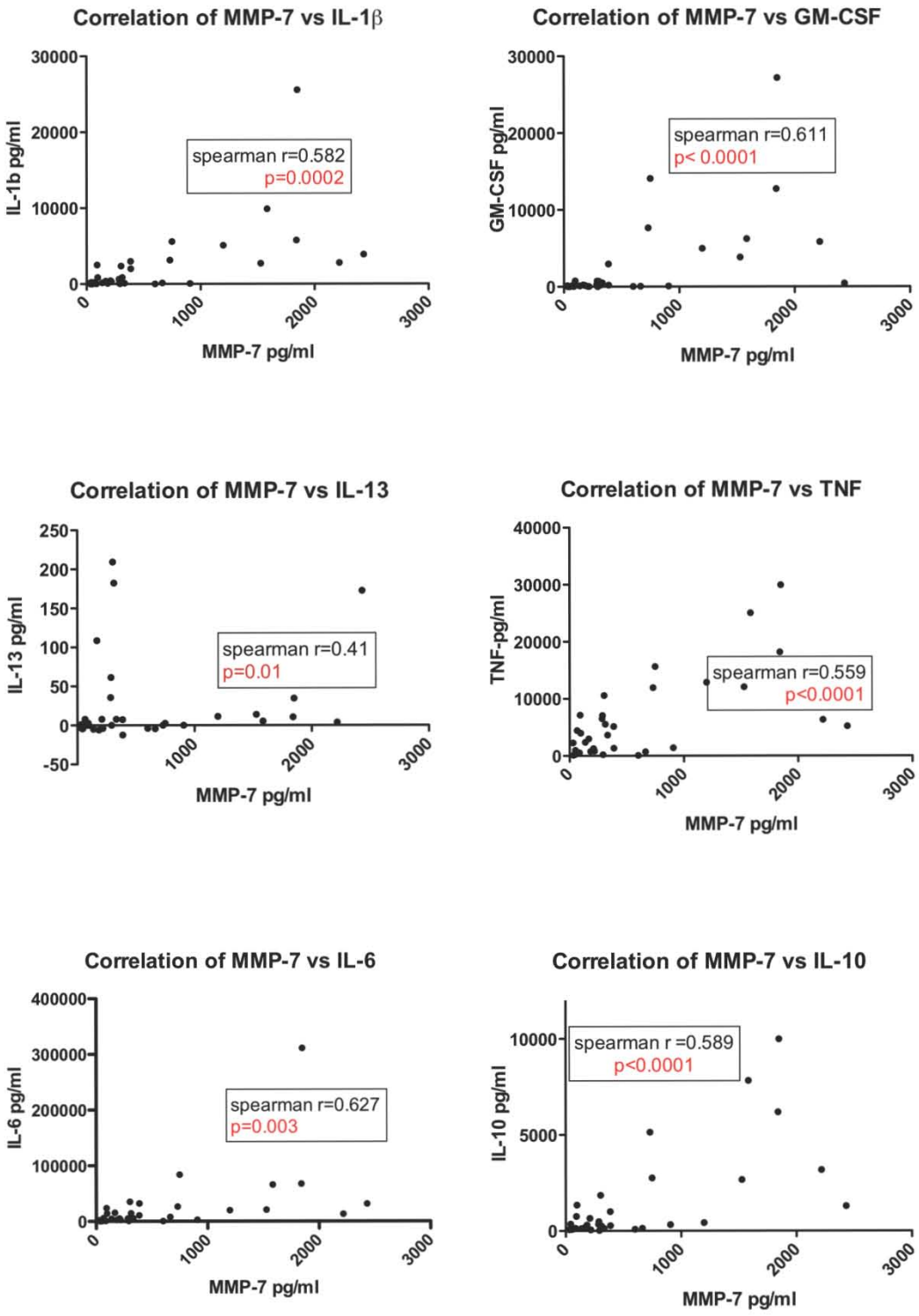


Figure 27 Correlation of MMP-7 vs. pro-and anti-inflammatory cytokines

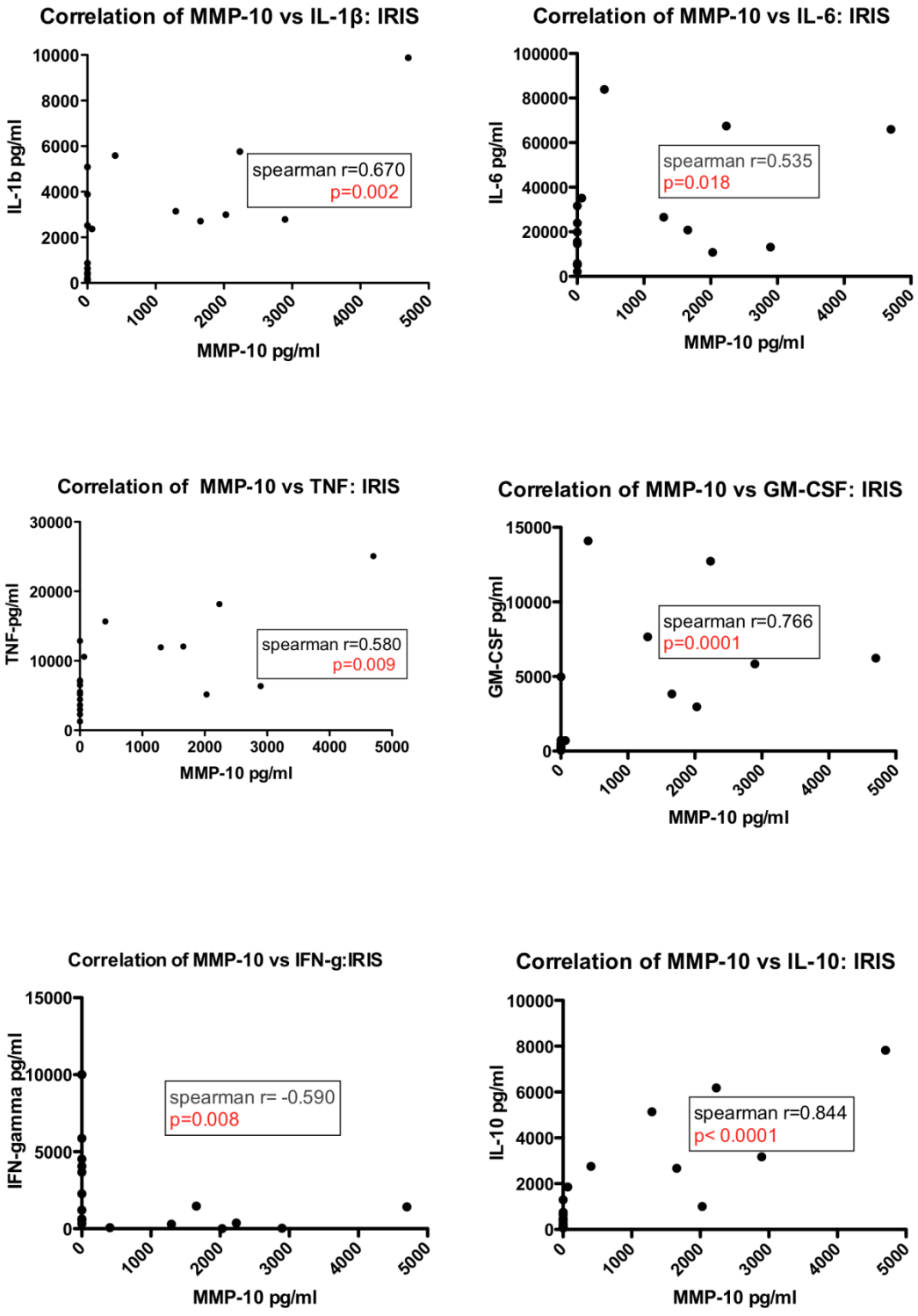


Figure 28 Correlation of MMP-10 vs. pro- and anti-inflammatory cytokines

5.2.2 **Correlation of MMPs with chemokines**

To assess the correlation between MMP chemokine expression MMP concentrations were correlated with the concentrations of IL-8, RANTES, MIP-1α, and MIP-1 beta. Figure 30 shows a summary of those correlations that were significantly different between MMPs and chemokines before corrections for multiple comparisons were made. The values in this figure were not corrected for multiple comparisons. There was a statistically significant positive correlation between between IL-8 and MMP-1, -2, -3, -7 and MMP-8 but not with MMP-10. When the concentration of MIP-1α was correlated with that of MMPs, a significant positive correlation was also observed with MMP-1, -3, MMP-7 and MMP-8. For MIP-1β and RANTES, no significant correlations were observed with any of the MMPs analysed.

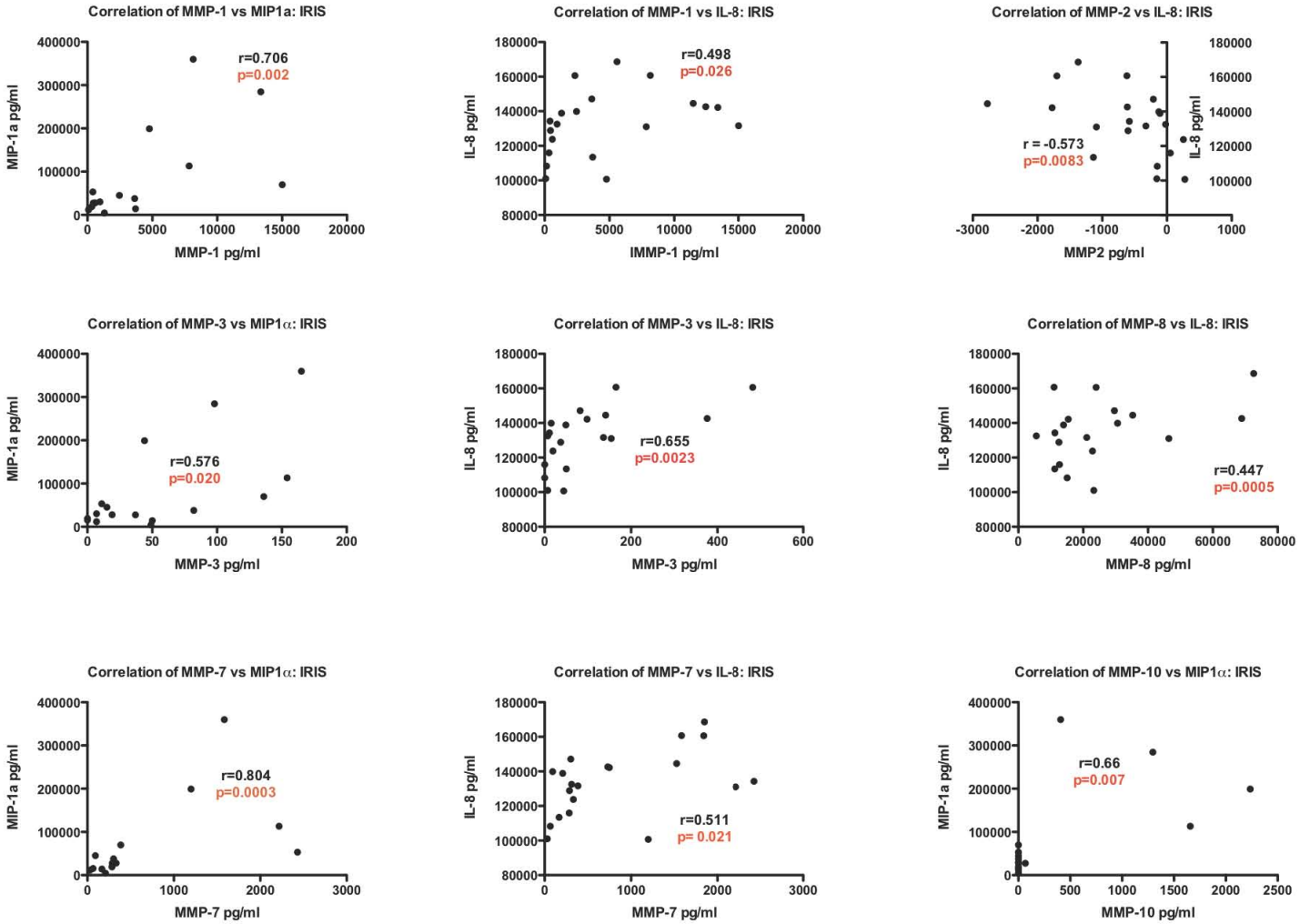


Figure 29 Summary of significant correlations between chemokines and MMPs

5.2.3 **Summary of multiple comparisons of correlations stratified IRIS vs. non-IRIS**

To further elucidate the significance of the statistical differences outlined in 5.2.1 and 5.2.2, I stratified the IRIS vs. non-IRIS patients and then corrected the p-values for multiple comparisons using the Bonferonni correction (multiplying the p values by n-1). Table 14 shows a summary of the stratified and corrected p-values. The positive significant correlations are highlighted in red while the blue highlighting shows the negative but significant correlations. After correcting for multiple comparisons the correlation between MMP-1 and IL-1β, IL-10 and MIP-1α remained statistically significant in the TB-IRIS group. MMP-2 correlated negatively to IL-10 and GM-CSF in IRIS patients and this remained statistically significantly but not with the other cytokines or chemokines. The correlation between MMP-3 and IL-1β, IL-6, IL-10, TNF and GM-CSF was statistically significant in IRIS patients even after multiple comparisons. MMP-3 showed significant correlation with IL-1β, TNF, and GM-CSF ($p<0.05$) in TB-IRIS after correction. Interestingly, MMP-7 even after multiple comparisons in the stratified datasets, there was a significant correlation between MMP-7 and IL-1β, IL-6, IL-10, GMC-SF, MIP-1α and IL-8. Similarly, MMP-10 showed significant correlation with many of the cytokines including IL-1β, IL-10, IL-13, GM-CSF and IL-8. MMP-10 was not expressed in the tissue culture supernatants of non-IRIS patients and is not shown in this summary table. It is interesting to note that while most of the correlations in this data were observed in TB-IRIS patients, even after multiple comparisons, many of these correlations remained significant in the TB-IRIS group and were practically absent in the non-IRIS controls.

Table 14 Summary of correalation p values stratified IRIS vs IRIS and then corrected for multiple comparison by Bonferonni correction (n-1)

		MMP-1	MMP-2	MMP-3	MMP-7	MMP-8	MMP-9	MMP-10
IL-1β	IRIS	0,033	0,330	0,022	0,001	5,610	4,620	0,022
	Non-IRIS	2,530	1,228	1,232	10,076	3,861	9,845	N/A
TNF	IRIS	0,209	0,396	0,011	0,055	3,652	5,940	0,099
	non-IRIS	0,286	5,555	0,781	8,228	7,821	6,237	N/A
IL-6	IRIS	0,143	0,088	0,033	0,033	3,377	4,917	0,050
	non-IRIS	0,693	4,807	0,132	8,162	6,413	5,753	N/A
IFN-g	IRIS	0,154	4,411	0,090	1,485	0,385	7,656	0,077
	non-IRIS	5,401	7,986	6,094	10,252	5,379	5,104	N/A
IL-2	IRIS	0,165	3,553	0,572	1,353	3,949	1,672	0,737
	non-IRIS	1,650	2,530	9,042	9,581	9,856	5,885	N/A
IL-10	IRIS	0,011	0,011	0,070	0,001	0,495	0,330	0,001
	non-IRIS	0,066	11,000	0,770	7,590	1,243	3,344	N/A
IL-12	IRIS	0,781	4,510	0,968	2,398	2,717	1,221	2,970
	non-IRIS	2,431	8,965	0,957	5,698	8,811	0,781	N/A
IL-13	IRIS	1,903	7,271	2,475	8,547	1,067	8,074	3,091
	non-IRIS	4,345	10,263	3,740	8,162	2,090	10,263	N/A
GM-CSF	IRIS	0,055	0,044	0,001	0,001	1,034	1,738	0,001
	non-IRIS	0,605	6,149	0,264	9,295	10,219	4,994	N/A
MIP-1α	IRIS	0,022	0,308	0,220	0,003	7,843	2,497	0,080
	non-IRIS	1,243	9,471	3,641	10,428	8,503	6,721	N/A
IL-8	IRIS	0,286	0,088	3,520	0,001	1,507	0,352	0,001
	non-IRIS	2,552	6,611	4,389	4,389	6,611	6,721	N/A

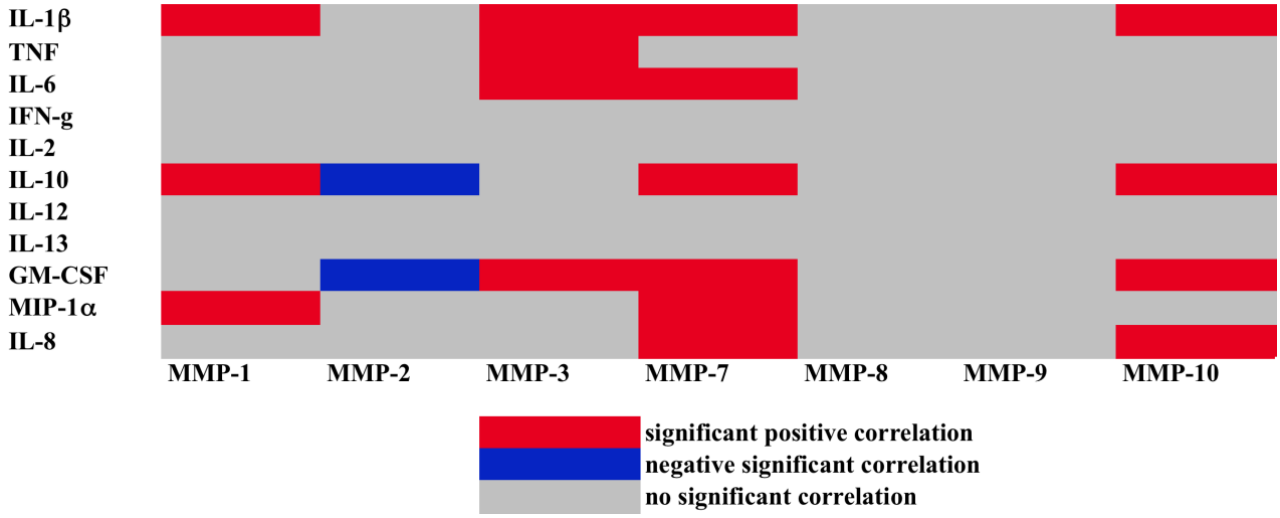


Figure 30 Schematic diagram showing the corrected p-values for correlations in IRIS

Figure 31 shows a schematic diagram summarising the Spearman correlation p values in IRIS patients between the different immune mediators after correcting for multiple comparisons. Positive significant correlations are highlighted while the negative significant correlations are highlighted in blue. The grey highlighting shows those correlations that were not significant. There were no significant correlations in non-IRIS (Table 14) hence, the non-IRIS correlations (p-values) are not show in this summary schematic diagram.

5.3 Discussion

In summary, the concentration of MMP-1, correlated significantly with IL-1β, IL-6, IL-10, IL-13, and TNF in the combined data sets. After stratification and correction for multiple comparisons, only IL-1β, IL-10 and MIP-1β remained significantly correlated with MMP-1. MMP-2 correlated significantly with IL-1β, MMP-2, IL-10, IL-6 and GM-CSF in the combined data sets. However, after stratifying IRIS versus non-IRIS and correcting for multiple comparisons, only IL-10 and GM-CSF in the IRIS group remained significantly correlated with MMP-2. The correlation of MMP-3 with IL-1β, TNF, IL-6, IL-10 and GM-CSF was significant in the combined data but was mainly emanating from the IRIS patients. This was shown after stratifying IRIS versus non-IRIS as only the correlations in IRIS were shown to be significant. There were no significant correlations observed between IL-12 and IFN-gamma and any of the MMPs either in IRIS or in non-IRIS patients.

MMP-7 and MMP-10 were significantly correlated to the cytokines IL-1β, GM-CSF, IL-13, TNF, IL-6 and IL-10. After correction for multiple comparisons, MMP-7 and MMP-10 remained consistently correlated with the pro-inflammatory cytokine IL-1β in IRIS patients. When chemokines were correlated with MMP levels, MMP-7 and MMP-10 showed significant correlation with IL-8 in IRIS patients, while MIP-1α significantly correlated with MMP-1 and MMP-7 in the IRIS group. The macrophage inflammatory proteins are chemoattractant cytokines crucial for immune responses towards infection and inflammation and are produced primarily by macrophages (Ren, Guo et al. 2010). MIP-1β and RANTES, which was expressed at very high levels, did not appear to correlate with the MMPs that were analysed in these cultures. The results presented here show evidence that the co-expression of MMPs in TB-IRIS may be exacerbating the inflammatory response evident in this condition.

The upregulated expression of MMPs upon MTB stimulation corroborates earlier findings from other workers who have showed that MMPs are induced by *M. tuberculosis* in different immune cells including monocytes and macrophages (Elkington, Nuttall et al. 2005; Harris, Green et al. 2007; Green, Elkington et al. 2010) The positive correlations observed in many of the pro-inflammatory cytokines and MMPs analysed in this study support the presence of a feedback mechanism where elevated levels of cytokines may be driving MMP expression. In turn, cytokines to some extent are known substrates of MMPs, and thus prone to degradation or cleavage by MMPs as well as stimulating MMP expression. GM-CSF has been shown to enhance TNF and IL-1β stimulated secretion of MMP-9 by THP-1 cells (Zhang, McCluskey et al. 1998).

It has been previously suggested that inflammation is a result of dyregulation and over expression of MMPs without a balance in the endogenous inhibitors, TIMPs, although this has proven difficult to show experimentally (Price, Farrar et al. 2001; Price, Gilman et al. 2003). While levels of TIMP-1 were detected to be distinctly higher in TB-IRIS patients in this work (Chapter 4), there was no significant correlation between TIMP-1 and any of the cytokines analysed in this study.

While the normal tissues contain little MMP activities, the production of pro-MMP-1, pro-MMP-3, pro-MMP-7 and pro-MMP-9 has been shown to be enhanced by IL-1β that is secreted from activated macrophages and many other cells (Ito, Mukaiyama et al. 1996). Once secreted and activated, these MMPs can then control the activity of IL-1β. The findings in this analysis showed consistent and significant correlations of IL-1β with many of the cytokines in IRIS, even after correction for multiple comparisons. Thus, it is possible that the

inflammatory mediators produced in IRIS (IL-1β included) are driving the production of MMPs which, once activate, will in turn degrade IL-1β as a way of modulating inflammation.

TNF and IL-1β have bee previously shown to enhance the production of MMP-9 by monocytes but had no effect on MMP-1, while combinations of IL-1β, TNF and GM-CSF have been shown to induce the synthesis of MMP-1 and enhance TIMP-1 production (Zhang, McCluskey et al. 1998). Individually, these cytokines were noted to induce MMP-9. IFN-gamma, IL-4, and IL-10 have been shown to inhibit the production of MMPs (Birkedal Hansen 1993). MMP-9, which has been implicated in TB immunopathology, although detected in very high levels in these patients, did not correlate significantly with any of the cytokines that were analysed. Thus while MMP-9 has been implicated in tuberculosis, its involvement in the inflammation or modulation of the inflammation associated with TB-IRIS does not seem apparent, form this data, but may be contributing to immunopathology in other ways.

The biological significance of MMP-2 (gelatinase B) on IL-1β degradation is still unclear and the results presented in this chapter also did not show any significant association between MMP-2 and IL-1β. However, MMP-2 correlated negatively and significantly with several cytokines and after comparison for multiple comparisons, IL-10 and GM-CSF remained significantly correlated in the IRIS group. This may imply that MMP-2 could be involved in immunomodulation. The significance of this however remains vague as no significant differences were observed between IRIS and non-IRIS when MMP-2 was analysed (chapter 4).

While these *in vitro* analyses have shown significant correlations between MMPs and cytokines, it would be of interest to further investigate if these relationships and expression levels were present in site of disease samples such as Bronchoalveolar Lavage (BAL). This could improve our understanding on the interaction between MMPs and TIMPs in inflammatory conditions such as TB-IRIS. The use of MMP inhibitors could also clarify the involvement of MMPs in this condition. Further more, *in vitro* assays such as tissue culture experiments could clarify and enhance our understanding on the involvement of MMPs in regulating inflammation in TB-IRIS. In summary, the analyses presented in this chapter demonstrate an association between the high levels of tissue degrading enzymes, MMPs and inflammatory mediators observed in these studies suggesting a potential role for MMPs in promoting or regulating inflammation in the inflammatory condition, TB-IRIS.

6 CHAPTER 6: ROLE OF IL-10 RELATED FAMILY OF CYTOKINES IN TB-IRIS

6.1 INTRODUCTION

The Interleukin-10 related family of cytokines comprises of cytokines structurally related to IL-10. These include IL-19, IL-20, IL-22, IL-24, IL-26 (AK155), IL-28 and IL-29 in humans in addition to some herpesviral and poxviral members (Fickenscher, Hor et al. 2002). Cytokines in this group are highly pleiotropic and are linked together mainly through genetic similarity and intron-exon gene structure (Commins, Steinke et al. 2008). The IL-10 related cytokines were identified in database searches for potential IL-10 homologs, and so far little is known about their biological activities of (Fickenscher, Hor et al. 2002) although suggestions have been put forward that they may participate in T-cell mediated diseases by a distinct regulation of T cell cytokine profiles (Mege, Meghari et al. 2006). One major similarity among these cytokines is the sharing of signalling receptors and having conserved signalling cascades (Fickenscher, Hor et al. 2002). Figure 32 below shows a summary of the relationship between the various signalling receptor subunits of the IL-10 homologs.

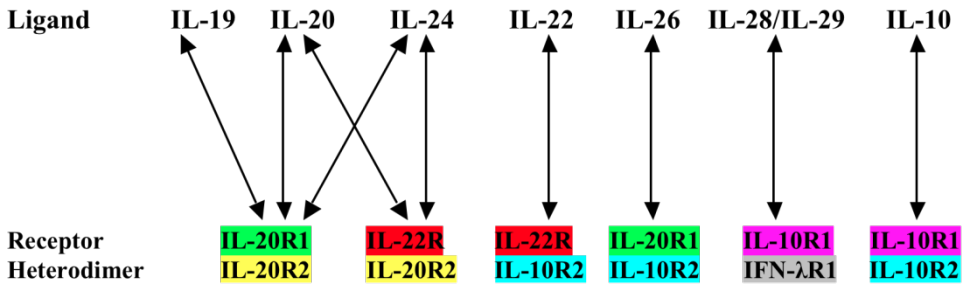


Figure 31 Summary of signalling receptors for IL-10 related homologs

Human IL-10 is the most well characterised molecule in this group with multiple biological effects including immunoregulatory and anti-inflammatory effects on different cell types (Saraiva and O'Garra 2010). The role of IL-10 as a survival, differentiation and proliferation factor for B cells and antibody production has also been described. IL-10 functions mainly to regulate Th1 cytokines, MHC class II and B7 molecules and the expression of co-stimulator molecules on macrophages that are necessary for optimal T-cell activation (Donnelly, Dickensheets et al. 1999; Fickenscher, Hor et al. 2002; Commins, Steinke et al. 2008). By inhibiting expression of these molecules on antigen presenting cells (APC), IL-10 directly suppresses the activation of T-cells and production of T-cell derived cytokines such as IFN-gamma and IL-2 (Donnelly, Dickensheets et al. 1999). Various immune cells produce IL-10 among them, activated monocytes, T cells (CD4+ and CD8+), macrophages and dendritic cells. The production of IL-10 is inducible by various stimuli and bacteria while gene expression may be delayed relative to other cytokines such as TNF and IL-1β (Donnelly, Dickensheets et al. 1999) and thus serves to dampen the inflammatory response by the suppression of inflammatory cytokines (Redpath, Ghazal et al. 2001). Growing evidence supports a modulating role for IL-10 in human allergic disease (Commins, Steinke et al. 2008).

Similar to IL-10, the other members of the IL-10 family are secreted as α– helical proteins whose amino-acid sequences are up to 30% identical to that of IL-10 with the encoding genes being located on two clusters in the human genome (Dumoutier, Van Roost et al. 2000; Wolk, Kunz et al. 2002). However, the knowledge of the biology of many of these IL-10 homologs remains incomplete. IL-19 is primarily produced by monocytes (Commins, Steinke et al. 2008), has been implicated in psoriasis and allergic disorders such as asthma (Liao, Cheng et al. 2004), and its expression can be induced by LPS, IL-4 and GM-CSF. IL-20 on

the other hand, appears to be the more divergent member of the group being produced mainly by monocytes and keratinocytes (Wolk, Kunz et al. 2002). Potential roles of IL-20 have been found in atherosclerosis, rheumatoid arthritis and angiogenesis with such patients expressing higher levels of IL-20 (Commins, Steinke et al. 2008). IL-24 was originally identified as a tumour suppressor molecule melanoma differentiation-associated gene 7 (mda-7) that was expressed in the melanocytes of healthy volunteers (Jiang, Lin et al. 1995). The majority of human cancer derived cell lines, including melanoma cells have been shown to undergo apoptosis upon exposure to IL-24 (Chada, Sutton et al. 2004). IL-26 has been shown to be generated mainly by CD4+CD45RO+ T cells and Natural Killer (NK) cells (Fickenscher, Hor et al. 2002) and is considered to be important in the transformation of human T-cells after their infection with Herpes saimiri virus. IL-28 and IL-29 (alternatively named IFN-λ1 and IFN-λ3 respectively) have some common features with the type 1 interferons including antiviral, anti-proliferative and antitumor activities (Fickenscher, Hor et al. 2002). While these genes have significant amino acid homology with type 1 interferons, their intron-exon structure more closely resembles that of IL-10, hence their classification into this group.

IL-21, although not a member of the IL-10 family, is a potent regulator of immune cells primarily expressed in activated CD4+ T cells (Parrish-Novak, Dillon et al. 2000). IL-21 has been tried as a therapy to alleviate allergic responses and was shown to successfully decrease T cell produced pro-inflammatory cytokines and mitigates allergic responses in mice studies. IL-21 may also be a critical factor in the control of persistent viral infections, including HIV infection (Hiromura, Kishida et al. 2007; Iannello, Boulassel et al. 2010) where it has been shown to improve the HIV-specific cytotoxic T cell responses and NK cell functions (White, Krishnan et al. 2007; Iannello, Boulassel et al. 2010).

IL-22 (IL-10 related T-cell derived inducible factor, IL-TIF) is a pro-inflammatory cytokine which plays an important role in innate pathogen defence (Dumoutier, Van Roost et al. 2000; Wolk, Kunz et al. 2004). More recently, studies in mice have shown that IL-22 is a Th17 cytokine (Liang, Tan et al. 2006) findings which however, have not been confirmed in human studies (Scriba, Kalsdorf et al. 2008). Expression of IL-22 has been shown to be highest in activated memory CD4 T cells, and lowest in activated natural killer cells (Commins, Steinke et al. 2008), with little or no expression being observed in other immune cells (Wolk K, Witte E, Immunity 2004). The preferential production of IL-22 by T cells suggests that increased expression of this molecule may exist in T-cell mediated diseases (Commins, Steinke et al. 2008; Wolk, Witte et al. 2009). IL-22 acts on non-immune cells and has been linked with severe inflammation in chronic T cell-mediated inflammatory diseases such as psoriasis, Chron's disease and rheumatoid arthritis (Wolk, Kunz et al. 2002; Wolk, Kunz et al. 2004; Hodge, Srinivasula et al. 2011). The link between IL-22 and severe inflammation suggests that IL-22 may contribute to the human anti-mycobacterial immune response.

The resolution of most infections is generally dependent on a host's ability to mount a protective immunoregulatory response to regulate the magnitude of inflammatory and immune responses. IRIS results from excessive pathological immune responses occurring during immune reconstitution in patients responding to cART. For this study, I hypothesized that IL-10 and its related homologs may have a role in the immune dysregulation that is associated with TB-IRIS.

6.2 RESULTS

6.2.1 Baseline characteristics of TB-IRIS patients versus non-IRIS controls

For this analysis, a subset of 20 paradoxical TB-IRIS patients versus 20 non-IRIS control patients was selected. Samples were well matched for gender distribution, biological age and baseline CD4 count. There were no significant differences between the two patient groups in terms of previous TB disease, TB disease form and median days of cART to IRIS onset (or sample collection in the case of non-IRIS controls). However, TB-IRIS patients had a significantly shorter duration between TB treatment and cART, ($p=0.028$). This is consistent with previous findings that have shown a shorter duration between TB- treatment and cART as one of the risk factors for developing TB-IRIS. Table 14 shows a summary of the baseline demographic and clinical characteristics for the patients analysed in this study.

Table 14 Baseline characteristics of 20 TB-IRIS versus 20 non-IRIS patients

	TB-IRIS	non-IRIS	p-value
n	20	20	N/A
Female n, (%)	(11) 55	12 (60)	0.27
Median age (years, IQR)	31.4 (23.2-45.7)	35.8 (22.2- 54.1)	0.273
Baseline CD4 count	56 (14.0- 193.0)	49.5 (5.0- 302)	0.59
Median days of TB-treatment Prior to cART	51.5 (14.0- 207.0)	78.0 (29.0- 173.0)	0.028
Median days of cART to IRIS onset or sample	14 (5.0-78.0)	14 (14-14)	0.94
Previous TB	5 (25)	4 (20)	1.00
TB Disease form n (%)			
Pulmonary TB only	10 (50)	16 (80)	0.096
Disseminated TB	10 (50)	4 (20)	0.096
Smear and culture positive n, (%)	10 (50)	13 (65)	0.52

6.2.2 **Analysis of IL-10 gene expression, *in vitro* and *in vivo* protein levels**

As previously reported in Chapter 3, IL-10 was highly expressed and was differentially higher in TB-IRIS patients at all levels of analysis (summarised in Figure 33). At the mRNA level, IL-10 was highly induced in TB-IRIS at 6 hours and even more so at 24 hours and this was statistically significant at both time points (p=0.04 and 0.0002 respectively). Secretion of IL-10 protein into corresponding tissue culture supernatants was also observed to be significantly higher in TB-IRIS compared to controls (p=0.012). Similarly, when *in vivo* secretion of IL-10 was determined in these patients by analysing serum samples, levels of IL-10 protein were shown to be much higher (more than a thousand fold) in TB-IRIS as compared to non-IRIS controls (p=0.0004). Thus, there were consistently higher levels of IL-10 in TB-IRIS compared to controls. To further explore the expression profiles the IL-10 related cytokines, IL-19, IL-21, IL-22, IL-24, IL-26, IL-27, IL28 and IL-29 mRNA transcript profiles were analysed.

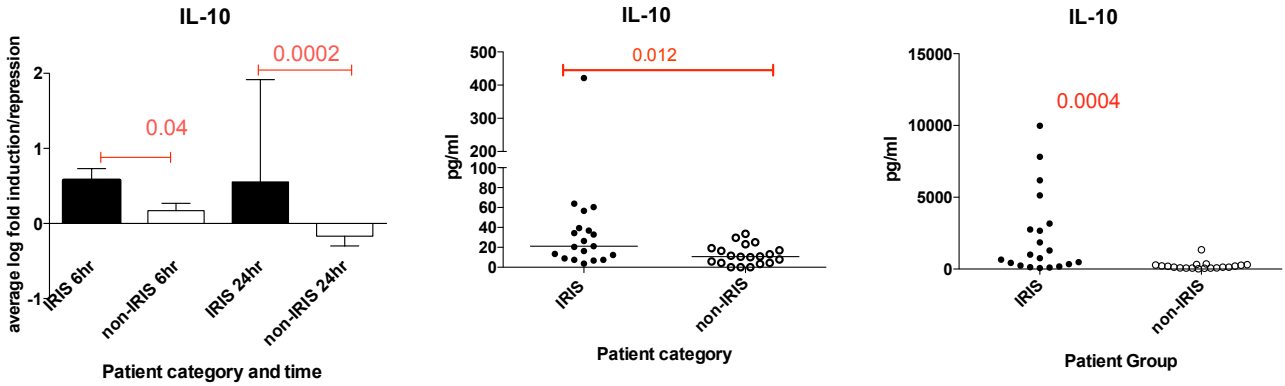


Figure 32 IL-10 log fold induction, supernatant and serum protein concentrations

6.2.3 **Transcript abundance of other IL-10 related genes analysed**

To further explore the expression profiles the IL-10 related homologs; IL-19, IL-22, IL-24, IL-26, IL-27, IL28 and IL-29 mRNA transcript profiles were analysed (Figure 34). IL-21, a regulatory cytokine was also included in this analysis. Transcript abundance was quantified by subtracting the cycle threshold (Ct) of beta-actin, the endogenous control, from the Ct of the gene of interest to obtain delta Ct (Table 16 and 17). At 6 hours, IL-22 transcript was significantly higher in IRIS than in non-IRIS patients in the simulated cultures. However, IL-24 transcript was significantly higher in non-IRIS controls than in TB-IRIS patients. Only IL-26 had significantly higher transcript in both the stimulated and unstimulated PBMC cultures of TB-IRIS patients. IL-28 transcript was marginally higher in non-IRIS patients and this difference was statistically significant. IL-29 transcript was basely detectable with most of the cycle thresholds being assigned the maximum value of 40 at both 6 and 24 hours.

At 24 hours, IL-20, IL-21 and IL-26 transcript levels were significantly higher in the stimulated cultures of TB-IRIS patients but not in the unstimulated cultures. However, IL-22 transcript levels were observed to be higher in non-IRIS in the unstimulated cultures, but increased significantly upon MTB stimulation ($p=0.015$). IL-28 transcripts were observed to significantly higher in non-IRIS at in the 24 hour unstimulated cultures but not so in the stimulated cultures. When comparing the effect of MTB stimulation on transcript levels in general, the transcripts of many of the IL-10 related genes increased significantly in both the IRIS and non-IRIS cultures upon MTB stimulation in the at both the 6 and 24 hour time points.

Table 15 Delta Ct values for IL-10 related cytokines genes after 6 hours of *in vitro* stimulation with heat killed *M.tb*

Median 24 hour delat CT values									p-values			
	unstimulated				stimulated				IRIS vs non-IRIS		unstim vs stim	
									unstim	stim	IRIS	non-IRIS
	IRIS	IQR	non-IRIS	IQR	IRIS	IQR	non-IRIS	IQR				
IL-19	11,7	2,3- 16,9	10,9	4,3 18,8	6,9	2,9-10,1	5,9	2,0-18,2	0,472	0,433	<0,0001	0,0002
IL-20	7,9	7,93-19,1	16,2	4,3-20,8	9,6	5,91-15,9	12,5	6,4-19,1	0,386	0,028	0,000	<0,0001
IL-21	14,3	8,1-18,3	14,9	4,3- 18	8,2	6,1- 14,9	10,1	6,9- 14,8	0,255	0,022	<0,0001	<0,0001
IL-22	18,3	12,4-19,9	15,5	12,5-19,7	12,0	6,8-14,9	14,7	8,0-19,2	0,004	0,015	<0,0001	0,199
IL-24	13,2	6,4-18,3	13,1	3,1- 14,0	10,4	5,5-15,1	9,9	12,9-15,1	0,944	0,661	0,007	0,020
IL-26	13,1	10,2-16-5	13,3	11,4-18,2	8,4	4,4-14,5	11,1	7,2-16,2	0,273	0,004	<0,0001	0,019
IL-28	10,7	8,6-14,2	9,7	7,5-11,8	10,2	6,4-12,3	10,2	8,3-11,9	0,012	0,609	0,034	0,025

Table 16 Delta Ct values for IL-10 related cytokines genes after 24 hours of *in vitro* stimulation with heat killed *M.tb*

	unstimulated				stimulated				p-values IRIS vs non-IRIS		unstim vs stim	
									unstim	stim	IRIS	non-IRIS
	IRIS	IQR	non-IRIS	IQR	IRIS	IQR	non-IRIS	IQR				
IL-19	13,4	5,5- 17,8	13,6	10,8- 19,2	8,8	6,5- 11,6	8,4	5,1- 12,1	0,935	0,804	<0,0001	<0,0001
IL-20	18,9	11,2- 21,3	18,4	9,6- 21,2	14,3	10,6- 19,8	23,2	8,5 18,8	0,236	0,103	<0,0001	<0,0001
IL-21	16,8	14,6- 20,9	16,3	10,9- 19,3	12,2	8,7- 16,4	13,9	8,5-18,1	0,705	0,070	<0,0001	<0,0001
IL-22	19,5	15,6- 21,6	18,6	13,8-21,3	13,0	8,3- 20,5	17,1	10,1-21,4	0,073	0,009	<0,0001	0,086
IL-24	14,1	7,6-20,8	13,3	5,8-18,3	11,8	7,7-15,7	9,5	5,9- 13,7	0,625	0,020	0,001	<0,0001
IL-26	16,9	14,4- 20	18,6	17,1- 21	16,4	11,3-19,4	17,5	13,0-20,7	0,008	0,042	0,002	0,010
IL-28	11,6	8,6-16,3	11,7	9,7-13,0	12,5	10,1-14,3	11,4	8,8-12,8	0,882	0,013	0,351	0,130

6.2.4 **Fold induction analysis of IL-10 related genes**

For many of the IL-10 related genes analysed, there was upregulation (as opposed to repression) of these genes in response to MTB stimulation (Figure 34). IL-19 and IL-20 were shown to be constitutively expressed as no differences were noted in the fold induction between stimulated and unstimulated samples and between IRIS and non-IRIS patients at both 6 and 24 hours. For IL-21, there was significant upregulation of the gene in TB-IRIS patients at 6 hours but not at the 24 hour time point ($p=0.0119$ and 0.759 respectively). IL-22 was the only gene that consistently showed significant upregulation at both 6 and 24 hours in TB-IRIS compared to non-IRIS 6 ($p=0.004$ and 0.0015 respectively). However for IL-24, IL-26 and IL-28 there were no significant differences observed between IRIS and non-IRIS at either time points. IL-28 and IL-29 were barely expressed in these cultures with IL-29 being marginally detectable 6 hours, but not detected in the 24 hour cultures (Figure 34).

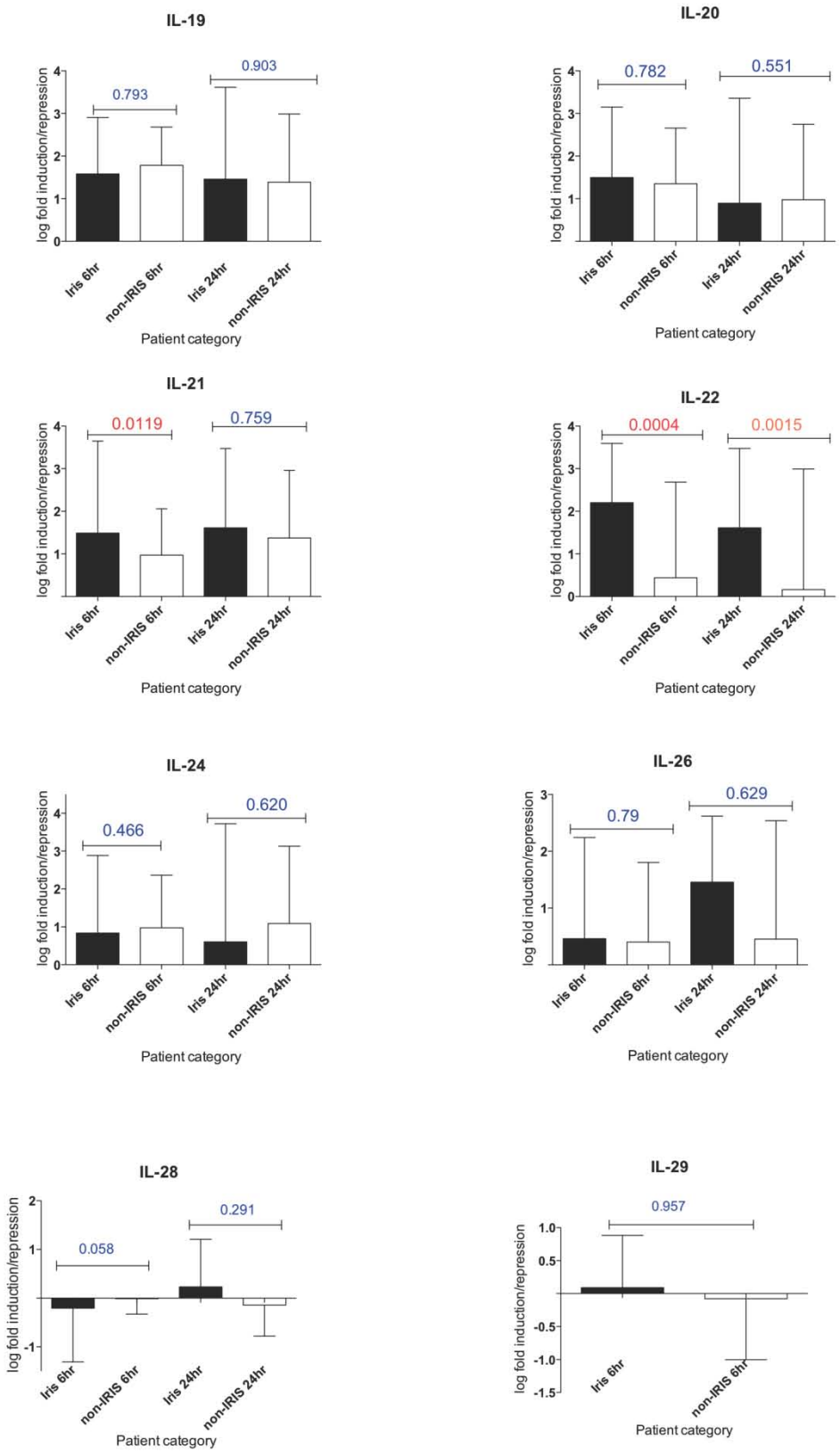


Figure 33 Fold induction analysis of IL-10 related cytokines

6.2.5 **Analysis of protein secretion into tissue culture supernatant and serum samples**

As a follow-up on the gene expression results, for those genes that showed significant differences in the protein mRNA levels, depending on availability of sample, protein levels were determined either in the tissue culture supernatants or serum samples or in both IL-19 protein secretion was determined in the corresponding tissue culture supernatants. IL-19 protein secretion in tissue supernatants was significantly higher level of IL-19 being detected in non-IRIS compared to IRIS patients upon MTB-stimulation (Figure 35). Follow-up analysis of IL-19 *in vivo* did not show any IL-19 protein in the serum samples with all values for the analysed samples falling below the detection limit of the assay for both patient groups.

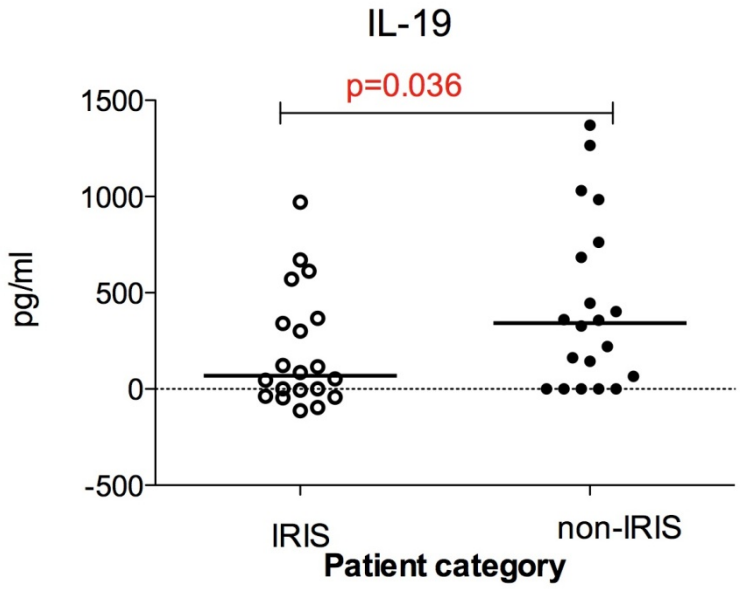


Figure 34 IL-19 concentrations in tissue culture supernatants

As a follow up on the IL-21 gene expression results obtained by quantitative RT-PCR, which showed significant fold induction of the IL-21 gene in TB-IRIS patients at 6 hour (figure 36), IL-21 protein was measured in the serum samples of TB-IRIS and non-IRIS patients. There

was no significant difference observed in the serum concentrations of IL-21 between IRIS and non-IRIS control patients in this experiment (Figure 36).

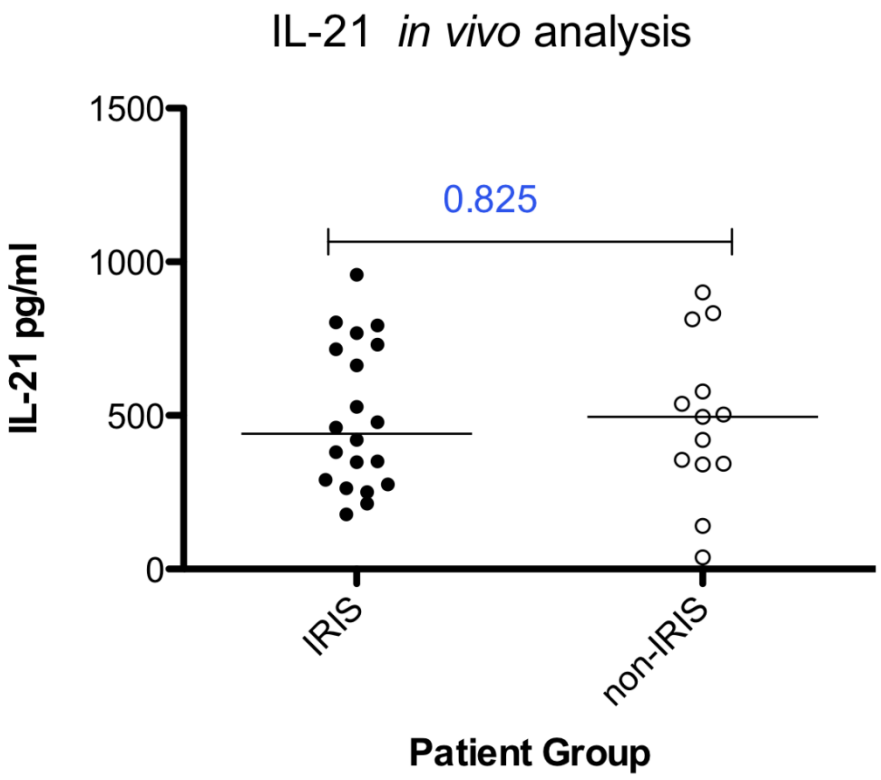


Figure 35 Serum (*in vivo*) concentrations of IL-21

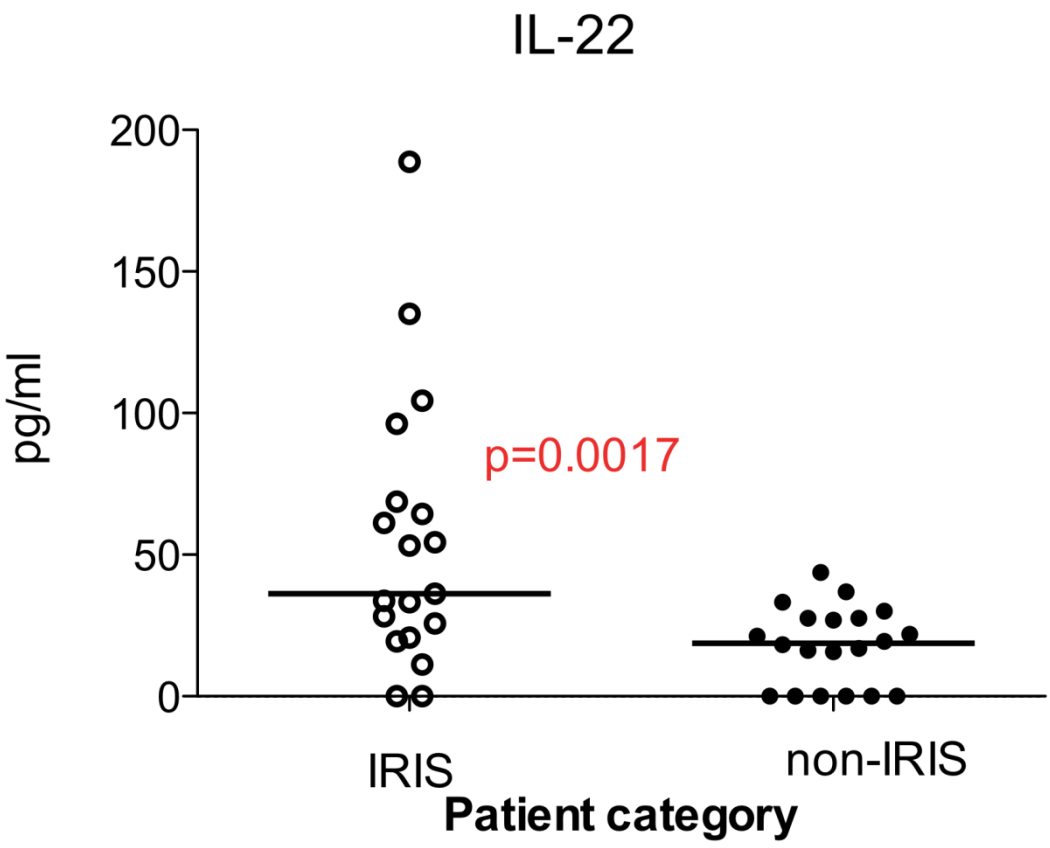


Figure 36 IL-22 concentrations in tissue culture supernatants

IL-22 secretion into tissue culture supernatants was not measured (due to inadequate sample availability) but was measured in the corresponding serum samples. Significantly higher levels of IL-22 were detected in the serum samples of TB-IRIS patients (p=0.0017) (Figure 37). Thus, IL-22 much like IL-10 showed consistent expression at different levels of analysis in these patients.

6.3 Discussion

In summary, analysis of the IL-10 related cytokines at different levels showed consistent and significant upregulation of IL-22 in TB-IRIS patients compared to non-IRIS controls. At gene expression level IL-22 fold induction was significantly higher in IRIS (p= 0.004 at 6 hours and p= 0.0015 at 24 hours), while IL-22 protein levels in serum were significantly higher in IRIS patients (p= 0.0017), much like the results observed for IL-10. IL-19 transcript tended to be marginally higher in non-IRIS patients and protein expression of IL-19 into tissue culture supernatant was significantly higher in non-IRIS patients (0.036). However, *in vivo* analysis of IL-19 did not show this differential expression with IL-19 protein being absent in the serum samples of either patient group. Apart from IL-22 and IL-19, none of the other IL-10 related genes (IL-20, IL-24, IL-26, IL-28 and IL-29) showed any differential expression between the two patient groups at any level of analysis. While IL-21 showed higher fold induction in IRIS at 6 hours, there was no significant difference between IRIS and non-IRIS control patients at 24 hours. Furthermore, analysis of IL-21 protein in the serum samples of these patients did not show any significant difference between the two patient groups (p=0.825).

While the regulatory role of IL-10 *in vitro* and in animal models is well documented, the role of this cytokine in clinical situations, particularly in infectious diseases is still the subject of intense investigation (Mege, Meghari et al. 2006). The production of IL-10 is regulated both at the transcriptional and translation level (Moore, de Waal Malefyt et al. 2001). However, production of IL-10 can also be stimulated by bacteria, viruses and parasites (Awomoyi, Marchant et al. 2002). Studies have now shown an association of IL-10 with increased susceptibility to infections caused by mycobacteria (Turner, Gonzalez-Juarrero et al. 2002). IL-10 has been shown to interfere with the innate and adaptive immune responses, thus

creating a favourable environment for the persistence of microbes, intracellular pathogens and chronic infections (Mege, Meghari et al. 2006). The viral versions of IL-10 are believed to assist in this immune evasion by tricking the immune system. The increased ability of macrophages to produce IL10 when stimulated with TLR ligands is also associated with an increased tendency to develop primary progressive TB (Saraiva and O'Garra 2010). The production of IL-10 has also been reported to be higher in patients who had active TB compared to TST responders (Vankayalapati, Wizel et al. 2003). In this study, measuring serum cytokine concentrations in *M. tuberculosis* infected patients, consistently high levels of IL-10 were observed in TB-IRIS, suggesting an involvement of IL-10 in the immunopathology of TB-IRIS (Vankayalapati, Wizel et al. 2003). TB-IRIS is a highly inflammatory condition. Thus it is also likely that the high levels of IL-10 observed in the blood of TB-IRIS patients in this study reflect an overspill of IL-10 from the sites of inflammation where IL-10 may be involved in regulating and resolving inflammation.

Recent studies in mice have reported that IL-10 deficient mice showed enhanced control of *M.tb* infection with significantly reduced bacterial loads (Redford, Boonstra et al. 2010), suggesting that IL-10 maybe suppressing the immune response to infection. However, the results of IL-10 studies in mice and in humans have been inconsistent to date and should be interpreted with caution. Other human studies have show that IL-10 may be associated with susceptibility to infections caused by fast growing mycobacteria (Muller, Aukrust et al. 1998). Thus, IL-10 may be contributing to the immunopathology that is evident in TB-IRIS patients. However, the mechanism by this happens is not clear and would require further investigation.

An association of IL-10 with HIV has been previously reported with significantly higher levels of circulating IL-10 being demonstrated in HIV-infected patients than in healthy controls (Stylianou, Aukrust et al. 1999). The highest levels of IL-10 were observed in patients with the most advanced clinical and immunological disease and virus load. IL-10 production has also been shown to increase in *M. avium* stimulated monocytes from HIV-infected patients, with the highest expression being observed in patients with advanced AIDS (Muller, Aukrust et al. 1998). Considering that the all of the patients investigated in this study were HIV-infected with the majority having very low CD4 counts (median 56 and 50 in TB-IRIS and non-IRIS respectively) it may be interesting to see if the high IL-10 levels observed in these patients correlated with HIV-1 viral load or clinical symptoms. However, routine viral load was not performed for these patients and could be addressed in future studies.

It is interesting that IL-22, like IL-10 was found to be significantly higher in TB-IRIS at both gene expression and serum protein levels. IL-22 has been previously implicated to have a role in T-lymphocyte disease, innate pathogen defence and in acute phase responses (Commins, Steinke et al. 2008) and has been associated with increased innate immunity in tissues (Wolk, Kunz et al. 2004) with expression being higher in outer body barriers such as skin, the respiratory and digestive system. The findings in this study suggest that IL-22 may be associated with the immunopathology in TB-IRIS. The co-expression of IL-10 and IL-22 as seen in this study has been investigated with suggestions that there may be interaction between these cytokines. However it has been shown despite having opposite effects (being anti-inflammatory and pro-inflammatory respectively) and sharing a signalling receptor subunit, IL-10 and IL-22 do not seem to interact as previously thought (Wolk, Witte et al. 2004).

While higher levels of IL-19 were observed in the tissue culture supernatants of non-IRIS patients, no IL-19 protein was detected in serum i.e. *in vivo*. IL-19, like IL-20 has been shown to be produced under inflammatory conditions and are thought to play an important role in the pathogenesis of some inflammatory diseases (Sabat, Wallace et al. 2007) although it remains unclear whether these cytokines regulate the function of immune cells. Recently, it has been suggested that IL-19, IL-20 and IL-24 could have potential roles in autoimmune diseases (Leng, Pan et al. 2011). However, from this study it remains unclear whether IL-19 and the other IL-10 homologs (IL-20, Il-24, IL-26, IL-28 and IL-29) have a role in TB-IRIS immunopathology.

Although not a member of the IL-10 super family, IL-21 has potential regulatory effects on cells of the immune system, including natural killer (NK) cells and cytotoxic T cells (Parrish-Novak, Dillon et al. 2000; Day, Abrahams et al. 2011). Expression of IL-21 has been shown to be higher in activated human CD4 T cells but not in most other tissues with Th17 cells producing higher levels of IL-21 than Th2 cells (Korn, Bettelli et al. 2007). IL-21 has also been implicated in auto-immune disease models and in the control of persistent viral infections (Nurieva, Yang et al. 2007; White, Krishnan et al. 2007). In this study, although higher IL-21 fold induction was detected at 6 hours in IRIS patients, this was not confirmed in vivo. Thus, whether IL-21 has a significant biological role in TB and in TB-IRIS may require further investigation.

The role of the other IL-10 related family cytokines has not been previously studied in TB or in TB-IRIS. Thus this work contributes substantial novelty to this subject. Considering the heterogeneity within this family of cytokines, it is likely that IL-20, IL-24, IL-26, IL-28 and IL-29 may be not have a clear role in this condition as shown by the findings reported here.

In summary, IL-10 and IL-22 but not IL-20, IL-24, IL-26, IL-28 and IL-29 appear to have a role in contributing to the immunopathology in TB-IRIS.

7 CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

While the clinical presentation of TB-IRIS is highly heterogeneous, diagnosis is mainly clinical and mainly relies on exclusion of alternative diagnosis. Thus, the study of the immunopathogenesis of TB-IRIS is important in enhancing understanding of the condition and could give insight into protective and pathogenic immune reconstitution. Thus, in addition to enhancing our understanding of the immunopathogenesis of TB-IRIS, studies of this nature could lead to the identification of biomarkers, which in turn may help in earlier prediction and diagnosis of the condition. This may also inform improved treatments and interventions to modulate TB-IRIS.

To determine whether dysregulated cytokine responses may be playing a role in the immunopathology of TB-IRIS, I conducted a case control analysis of paradoxical TB-IRIS patients vs. non-IRIS control patients who were on similar treatment but did not develop TB-IRIS. Stimulation of PBMC with *M.tuberculosis* increased the transcript abundance and concentrations of multiple cytokines *in vitro* for TB-IRIS vs. non-IRIS control patients. Follow-up analysis of those cytokines that were differentially upregulated *in vitro* in the corresponding serum samples i.e. *in vivo* demonstrated higher concentrations of the pro-inflammatory cytokines IL-6, TNF and IFN-gamma in TB-IRIS patients. Comparison of prednisone vs. placebo treated patients showed a significant decrease of both IL-6 and TNF but not IFN-gamma in prednisone treated patients. The elevation of many pro- and anti-inflammatory cytokine transcripts and protein levels shown in this study strongly suggests that cytokine release contributes to the immunopathology and symptoms in TB-IRIS.

While the levels of cytokines observed in this study may not be as high as those reported in the TGN1412 trial (Suntharalingam, Perry et al. 2006) or in H5N1 infection (de Jong,

Simmons et al. 2006) it is evident from these findings that the exaggerated cytokine expression observed in TB-IRIS contributes substantially to immunopathology in this condition. TNF has consistently been associated with both protection and pathology in TB (Bekker, Maartens et al. 1998; Keane, Gershon et al. 2001). While recent studies have documented a beneficial effect of TNF blockade on paradoxically deteriorating TB in HIV-1 uninfected patients, the potential use of this therapy in TB-IRIS patients presents challenges as could potentially lead to the reactivation of latent TB (Keane, Gershon et al. 2001).

The use of prednisone therapy in TB-IRIS cases has been shown to result in more rapid symptom resolution in TB-IRIS cases as compared to placebo treated patients (Meintjes, Wilkinson et al. 2010). It is interesting to note that, in keeping with these clinical findings, prednisone treatment in TB-IRIS patients significantly downregulated the levels of TNF and IL-6. Taken together, these results suggest that prednisone therapy modulates the pro-inflammatory cytokine response characteristic of TB-IRIS. Thus, short-term adjunct prednisone therapy is potentially a useful intervention in TB-IRIS. A phase II study in patients with know drug sensitive TB undergoing severe TB-IRIS in whom cortecosteroid therapy was either ineffective or contraindicated may be justifiable.

While the findings on the potential modulation of TNF and IL-6 by prednisone in TB-IRIS are insightful, caution should be exercised in translating these findings to a clinical trial. It may also be prudent to confirm these findings in a larger sample size. Similarly, the results of stratification of IRIS patients on the basis of severity showed a trend towards lower cytokine levels in patients who had less severe IRIS. However this study was underpowered and so significant conclusions on the association of cytokine levels and TB-IRID severity. Further work, possibly with larger samples would be recommended in order to validate these

preliminary findings. While there has been work published to suggest that IL-7 could be used as a biomarker in HIV-TB patients who developed IRIS, (Hodge, Srinivasula et al. 2011), the results presented in this thesis did not confirm these findings. Further work on the possible association of IL-7 with and its potential use as a biomarker in TB-IRIS would be recommended.

The hypothesis that a deficiency of regulation may contribute to TB-IRIS has been previously put forward and is of considerable interest. Analysis of the anti-inflammatory cytokine, IL-10 showed significantly elevated levels of this immunoregulatory cytokine in TB-IRIS compared to non-IRIS control patients. IL-10 was consistently higher in TB-IRIS at mRNA, *in vitro* protein and *in vivo* protein concentrations. Further analysis of the gene expression and *in vivo* protein concentrations for the other IL-10 related cytokines showed IL-22 to be significantly more upregulated in TB-IRIS at mRNA level. Follow-up analysis of IL-22 *in vivo* showed that IL-22 protein expression in serum was significantly higher in TB-IRIS, a trend that was very similar to that observed for IL-10 hence suggesting a potential role of these two related cytokines in the immunopathology of TB-IRIS. The other IL-10 related homologs did not appear to have a clear role in TB-IRIS. However, this may be justifiable considering the substantial heterogeneity within the goup of IL-10 related cytokines. It would be interesting to investigate further whether the involvement of IL-10 and IL-22 in the immunopthology of TB-IRIS is via a similar mechanism.

TB-IRIS is typically characterised by matrix destruction and excessive immune-mediated tissue damage according to clinical observations. In this study, I hypothesised that MMPs may be playing a central part in this immunopathology (Elkington and Friedland 2006; Elkington, D'Armiento et al. 2011). My findings (Chapter 4) showed that *M. tuberculosis*

stimulation of PBMC differentially increased the transcript levels for multiple MMPs in paradoxical TB-IRIS patients. The up-regulation of MMP-1, -3, -7 and -10 by *M. tuberculosis* is consistent with previous reports in primary human macrophages, monocytes (Harris, Green et al. 2007; Elkington, Green et al. 2009) and PBMC. MMP-7 and MMP-10 remained differentially induced in TB-IRIS patients at both 6 and 24 hours. MMP-7 correlated closely with IRIS and was suppressed by prednisone treatment. While high levels of MMP-9 were detected, divergent MMP-9 gene expression or secretion between the TB-IRIS and non-IRIS patients was not demonstrated. These findings suggest that while MMP-9 may be generally associated with the pathogenesis of tuberculosis as observed in a *M.tuberculosis* model in zebrafish (Volkman, Pozos et al. 2010), it does not appear to be specifically associated with the immunopathology that is evident in TB-IRIS.

The biology of MMPs is still incomplete and is currently under intense investigation. While the available assays may measure pro-MMP (e.g. the MMP-10 assay), active MMP only (e.g. MMP-9 assay) or both the active and pro-enzyme forms (e.g. MMP-1), most of the available assays do not reliably measure all forms of the proteins available in a biological sample concurrently. This places a limitation on the analysis of MMPs as it means that not all biologically available protein can be accurately measured in a given sample. While blood is the most readily used biological sample for the study of the immunology of tuberculosis, admittedly it is not the best compartment and may not be representative of the immunological changes occurring at the site of disease. Ideally, MMPs would preferably be measured in site of disease samples or using tissue biopsies, samples that were not readily available for our study. The MMPs that filter through to serum may only be a spillover of what is produced at the site of disease. Hence the levels of these enzymes available in systemic circulation i.e. in the blood compartment (serum) may not always correspond to what is present at the site of

disease. To this end, it would be necessary to further investigate these findings in representative site of disease samples inorder to confirm whether the observations noted at the site of disease confirm our findings on the involvement of MMPs in TB-IRIS immunopathology. Studies of MMPs in BAL, sputum or tissue biopsies of TB-IRIS patients would be recommended in thi this regard.

Apart from degrading the extracellular matrix, MMPs have been shown to have other roles, including immunomodulation, and facilitating the generation of chemotactic gradients, thus acting on other non-matrix substrates including cytokines and growth factors (McCawley and Matrisian 2001). In this thesis, *in vitro* analysis of the concentrations of multiple MMPs analysed correlated significantly with the concentrations of several cytokines including IL-1 beta, TNF, IL-6 and GM-CSF in addition to IL-10 mainly in TB-IRIS patients. These findings further suggest that the co-expression of MMPs, cytokines and chemokines altogether associates strongly with the immunopathology in TB-IRIS. Further work on this, particularly in vitro assays or work I animal models may help to elucidate the mechanisms by which co-expression of MMPs and cytokines are involved in TB-IRIS immunopathology.

In conclusion, the immunopathogenesis of TB-IRIS appears to be mediated by the interaction of a variety of immune mediators including pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines and matrix metalloproteinases. The findings presented in this thesis also give strong evidence for the involvement of tissue degrading enzymes, MMPs in the immunopathology of paradoxical TB-IRIS. Whilst one of the largest studies of TB-IRIS to date, the series of studies presented in this thesis show comprehensive analyses which make substantial contributions, thus expanding our knowledge on the immunopathology of TB-IRIS. Here, I have comprehensively characterised the immunopathology of paradoxical TB-

IRIS. This work makes a significant contribution to knowledge and to existing literature on the subject and the study of TB-IRIS immunopathology. Novel findings are presented in this thesis showing in-depth immunological profiles associated with paradoxical HIV-TB-IRIS. The data presented in this thesis show distinct patterns with regards to the cytokines, matrix metalloproteinases, and the correlations showing potential interactions of these immune mediator molecules in association with TB-IRIS immunopathology.

MAIN OUTPUT FROM THIS WORK

The work presented in this thesis has been presented at various local and international Scientific meetings as detailed below:

POSTER PRESENTATIONS

1. "Pro- and anti-inflammatory cytokine mRNA profiling of the HIV-tuberculosis immune reconstitution inflammatory syndrome."

Rebecca Tadokera, Graeme Meintje, Katalin Wilkinson, Molebogeng Rangaka, Kerryn van Veen, Keira Skolimowska, Gary Maartens and Robert J Wilkinson. 5th IAS Conference on HIV Pathogenesis, Treatment and Prevention, Cape Town, South Africa, July 2008.

2. "Matrix Metalloproteinases in HIV-Tuberculosis associated Immune Reconstitution Inflammatory Syndrome."

Rebecca Tadokera, Graeme Meintjes, Keira Skolimowska, Katalin A. Wilkinson, Paul T. Elkington, Kerryn van Veen1, Ronnett Seldon, Gary Maartens and Robert J. Wilkinson. Overcoming the Crisis of TB and AIDS (T2-2010) Arusha International Conference Centre, Arusha, Tanzania, October 20 - October 25, 2009. *Received a Keystone Global Health Travel scholarship based on the abstract.

3. "Matrix Metalloproteinases in HIV-Tuberculosis associated Immune Reconstitution Inflammatory Syndrome."

Tadokera R, Meintjes GA, Wilkinson KA , Rangaka MX , Skolimowksa KH, K1, Rebe K , Chegou NN, Walzl G, Maartens G, and Wilkinson RJ. South African Immunological Society Conference 9-11 December 2009, Vineyard Hotel, Colinton Road, Newlands, Cape Town.

4. "Hypercytokinaemia accompanies HIV-associated TB-IRIS."

Tadokera R, Meintjes GA, Wilkinson KA , Rangaka MX , Skolimowksa KH, , Rebe K , Chegou NN, Walzl G, Maartens G, and Wilkinson RJ. 14th International Congress of Immunology, (ICI 2010). Kobe, Japan, 22-27 August, 2010. *Received a Travel scholarship for postgraduate students to attend the conference.

5. "Contribution of cytotoxic cells to antiretroviral induced pathological immunity in HIV associated tuberculosis (TB-IRIS)"

Tadokera R, Meintjes GA, Skolimowska KH, Matthews K, Nicol MP, Martens G, Rangaka MX , Rangaka MX, Wilkinson RJ. Wilkinson KA. Keystone Symposia Tuberculosis: Immunology, Cell Biology and Novel Vaccine Strategies. Vancouver, British Columbia, January 15-January 20, 2011. * Received an NIAD Scholarship to attend this conference

ORAL PRESENTATIONS ARISING FROM THIS WORK

1. "Pro- and anti-inflammatory gene expression and the HIV-TB immune reconstitution inflammatory syndrome."

Rebecca Tadokera, Graeme Meintjes, Katalin Wilkinson, Molebogeng Rangaka, Kerryn van Veen, Keira Skolimowska, Gary Maartens and Robert J Wilkinson. MLW Research Conference, Club Makokola, Mangochi, 30th August 2009 - 2nd September 2009. * Received a CIDRI scholarship to attend the conference based on the abstract.

2. "Exaggerated cytokine release and the HIV-tuberculosis immune reconstitution inflammatory syndrome."

Rebecca Tadokera, IIDMM Immunology Seminar Series, Wolfson Pavillion, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, 9 June 2010.

3. "Exaggerated cytokine release and the HIV-TB associated immune reconstitution inflammatory syndrome."

Rebecca Tadokera, Graeme Meintjes, Katalin Wilkinson, Molebogeng X Rangaka, Kerryn van Veen, Keira Skolimowska, Gary Maartens and Robert J Wilkinson. UCT/GSH Annual Medical Research Day, GHS, Cape Town, 30 September 201

4. "MMP-7 and tissue damage in HIV associated TB-IRIS"

Rebecca Tadokera, Graeme Meintjes, Katalin A Wilkinson, Keira H Skolimowska, Naomi Walker, Jon S Friedland, Gary Maartens, Paul T G Elkington, Robert J Wilkinson. CIDRI and Imperial College Wellcome Centre Annual Scientific Meeting. IIDMM, University of Cape Town, 12-14 March 2011

PUBLICATIONS ARISING FROM THIS WORK

1. Part of the work presented in Chapter 3 was published in the European Respiratory Journal, September 2010 (epub ahead of print) : Hypercytokinemia characterizes antiretroviral-induced pathological immunity in HIV-associated tuberculosis (TB-IRIS).

Rebecca Tadokera, Graeme Meintjes, Keira H Skolimowksa, Katalin A Wilkinson, Kerryn Matthews, Ronnett Seldon, Novel Chegou, Gary Maartens, Molebogeng Xheeda Rangaka ,Kevin Rebe, Gerhard Walzl, Robert J Wilkinson

2. Part of the work presented in Chapter 4 has been submitted and is under review:

Matrix metalloproteinase-7 and tissue damage in paradoxical HIV-Tuberculosis associated Immune Reconstitution Inflammatory Syndrome.

Rebecca Tadokera, Graeme A Meintjes, Katalin A Wilkinson, Keira H Skolimowska, Naomi Walker, Jon S Friedland, Gary Maartens, Paul T G Elkington, Robert J Wilkinson

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9 APPENDICES

Appendix 1: Summary of Cytokine and MMP genes analysed, assay ID numbers and the primer amplicon lengths

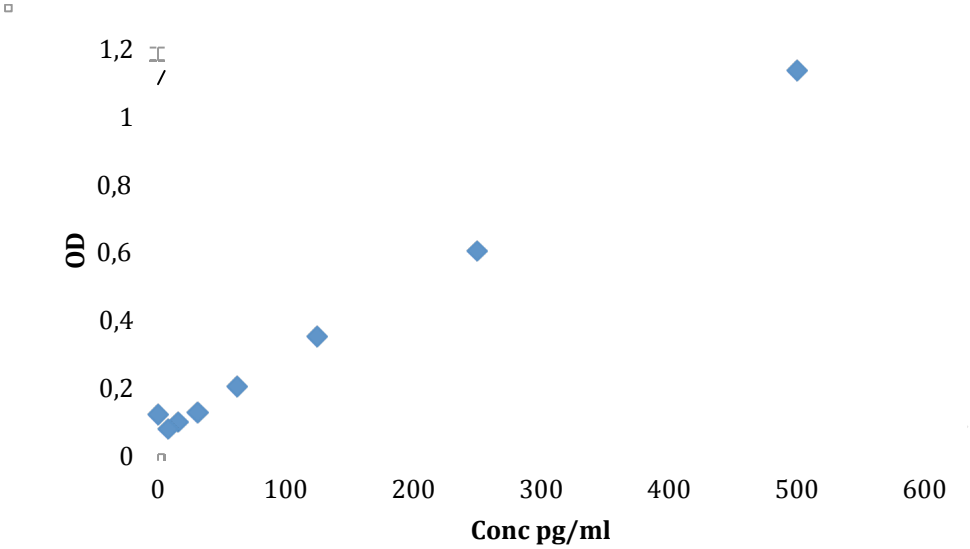
Molecule	Gene name	Assay ID Details	Amplicon Length
IL-1β	interleukin 1-beta	Hs00174097_m1	94
IL-2	interleukin 2	Hs00174114_m1	94
IL-4	interleukin 4	Hs00174122_m1	70
IL-5	interleukin 5	Hs00174200_m1	87
IL-6	interleukin 6	Hs00985639_m1	66
IL-7	interleukin 7	Hs00174202_m1	75
IL-8	interleukin 8	Hs00174103_m1	101
IL-9	interleukin 9	Hs00174125_m1	92
IL-10	interleukin 10	Hs00174086_m1	119
IL-12p40	interleukin 12p40	Hs01011518_m1	72
IL-13	interleukin 13	Hs00174379_m1	82
IL-15	interleukin 15	Hs00542562_m1	67
IL-17A	interleukin 17a	Hs00174383_m1	80
IL-18	interleukin 18	Hs01038788_m1	115
IL-19	interleukin 19	Hs00604657_m1	68
IL-20	interleukin 20	Hs00218888_m1	78
IL-21	interleukin 21	Hs00222327_m1	84
IL-22	interleukin 22	Hs00220924_m1	68
IL-23A/IL-23p19	interleukin 23 alpha subunit	Hs00413259_m1	53
IL-24	interleukin 24	Hs01114274_m1	67
IL-26	interleukin 26	Hs00218189_m1	81
IL-27	interleukin 27	Hs00377399_m1	75
IFN-γ	interferon gamma	Hs00174143_m1	79
CSF2/GMCSF	Granulocyte macrophage colony stimulating	Hs00171266_m1	113
TNF	Tumour necrosis factor	Hs00174128_m1	80
TGF-β1	Transforming growth factor	Hs00171257_m1	63
IL-27A/IL-27p28	interleukin 27	Hs00377366_m1	75
IL-28	interleukin 28	Hs00820125_g1	53
MMP-1	matrix metallopeptidase-1	Hs00899658_m1	64
MMP-2	matrix metallopeptidase-2	Hs01548727_m1	65
MMP-3	matrix metallopeptidase-3	Hs00968308_m1	98
MMP-7	matrix metallopeptidase-7	Hs01042795_m1	101
MMP-8	matrix metallopeptidase-8	Hs01029057m_1	67
MMP-9	matrix metallopetidase-9	Hs00957555_m1	79
MMP-10	matrix metallopeptidase-10	Hs00233987_m1	82
MMP-11	matrix metallopeptidase-11	Hs00968295_m1	60
MMP-12	matrix metallopeptidase-12	Hs00159178_m1	62
MMP-13	matrix metallopeptidase-13	Hs00233992_m1	91
TIMP-1	metallopeptidase inhibitor 1	Hs99999139_m1	55
TIMP-2	metallopeptidase inhibitor 2	Hs00234278_m1	73
MIP-1α (CCL3)	Macrophage inflammatory protein-1	Hs00234142_m1	53
MIP-1β (CCL4)	Macrophage inflammatory protein-2	Hs99999148_m1	94
RANTES	Chemokine motif Ligand 5	Hs00174575_m1	63

Appendix 2: Preparation of Solutions and Buffers for Gelatinase Zymography

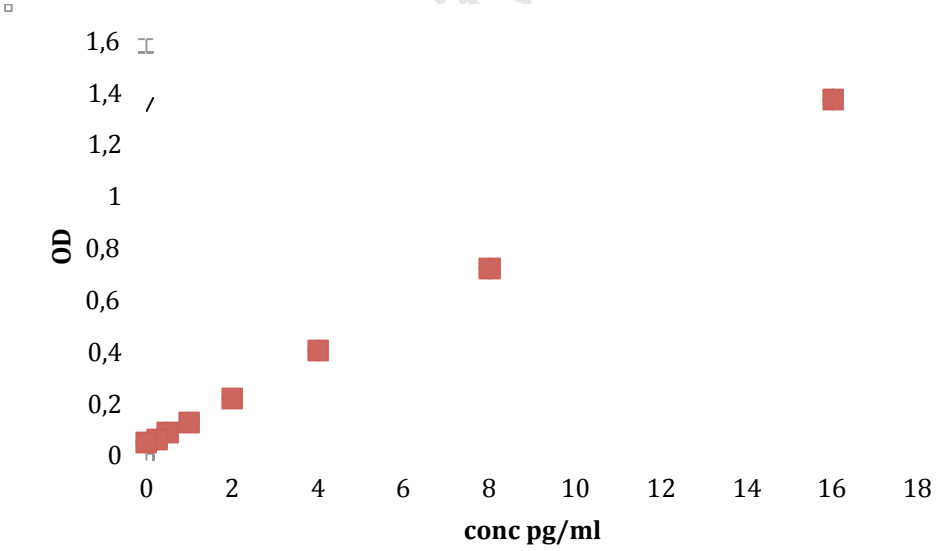
Solution/Buffer	Ingredients	Recipe/Supplier
1.2% gelatin solution	1.2g gelatin 100 ml Deionised water	Merck 1.04070 Mixture was heated in a 65°C water bath for 10 minutes and aliquots stored at 4°C.
11% Running gel (for 2 gels) 15cm x 15cm x 1mm	40% acrylamide 20 mls Running gel buffer 30 mls Deionised H_2O 8 mls 1.2% gelatin 160µl TEMED 320 µl 10% (w/v) APS	Merck 1.00641 Merk 1.04070 Merck 1.10732 Merck 1.01201 Stock made up and frozen as 1ml aliquots for single time use. TEMED and APS were added just before pouring the gel.
Stacking gel (for 2 gels) - 5% acrylamide	2.5mls 40% acrylamide 5mls stacking gel buffer 12.5mls distilled H_2O 40µl TEMED 80µl 10% (w/v) APS	Merck 1.00641 Merck 1.10732 Merck 1.01201
Running buffer (10x stock) Running buffer (1x working solution)	30.28g Tris base 144.12g glycine 10ml 10% (w/v) SDS 100ml stock as above	Sigma-Aldrich T4661-1KG Sigma L4509 Add to 1L distilled water Sigma L6026 Make up to 1L with distilled water
Sample loading buffer (5x)	5mls 0.5M Tris HCl (pH 6.8) 5ml Glycerol 0.5g SDS Crystals of Bromophenol	Sigma T6666 Sigma G6279 Sigma L6026

	blue	
MMP-9 standard buffer	**Tris-HCl 2.36g 60mM** **250mls distilled water** **$CaCl_2$ 0.55g 15mM** **NaCl 1.17g 80mM** **BSA 0.1% 0.25g**	**Make up in 250mls distilled water and freeze as 0.5ml aliquots** **Merck 1.02378** **Sigma Aldrich S5886-1KG** **Sigma A9418-100G**
2.5% Triton X-100 renaturing buffer	**25mls Triton X-100** **1L De-ionised water**	**Merck 648466**
Low salt collagenase buffer (10x) **1x working solution**	**60.6g Tris base MW 121g** **117g NaCl MW 58** **5.5g $CaCl_2$ MW 111g** **670µl Brij-35 (30% w/v)** **100mls 10x stock,** **900mls deionised water**	**Sigma-Aldrich T466-1KG** **Sigma Aldrich S5886-1KG** **Sigma Aldrich S5670** **Make up to 900mls with deionised water** **Adjust pH to 7.6 with conc. HCL and top up to 1L** **Check and adjust pH to pH if required**
Destain solution	**200mls glacial acetic acid** **600mls methanol** **1200ml distilled H_20**	**Make in 1200ml distilled water. Store at RT**
0.2% Coomassie blue (stock solution) **Coomassie blue (working solution) – sufficient for 2 gels**	**1 tablet PhastGel-Blue-R Coomassie blue tablets** **80mls distilled water** **10ml of stock solution** **190mls of destain solution.**	**Pharmacia, Sweden, GE Healthcare 17-0518-01** Stir for 10 minutes. Add 120mls methanol. Filter (Whatman No. 1 filter paper). Stored at 4^0C

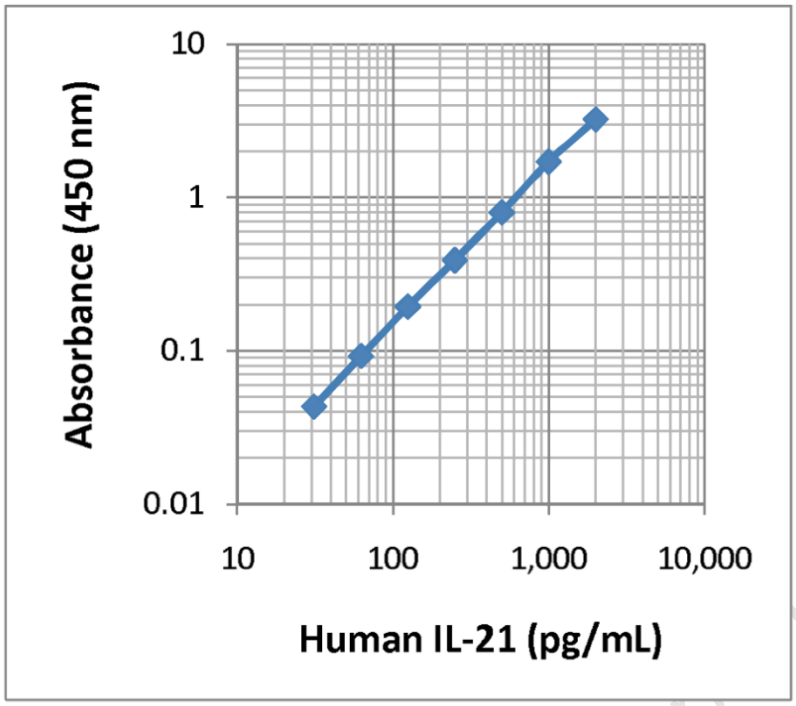
Appendix 3: IL-17 standard curve



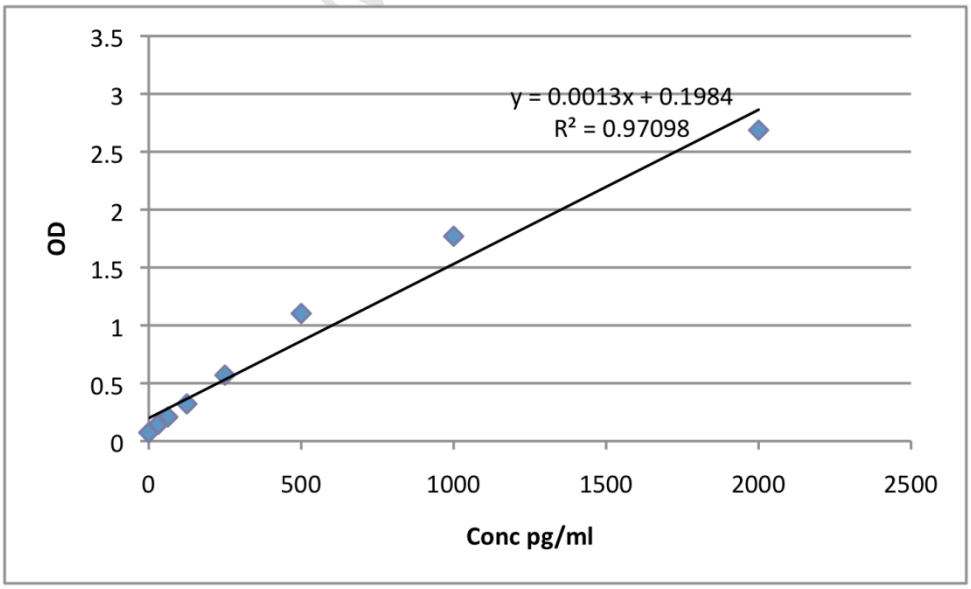
Appendix 4: IL-7 standard curve



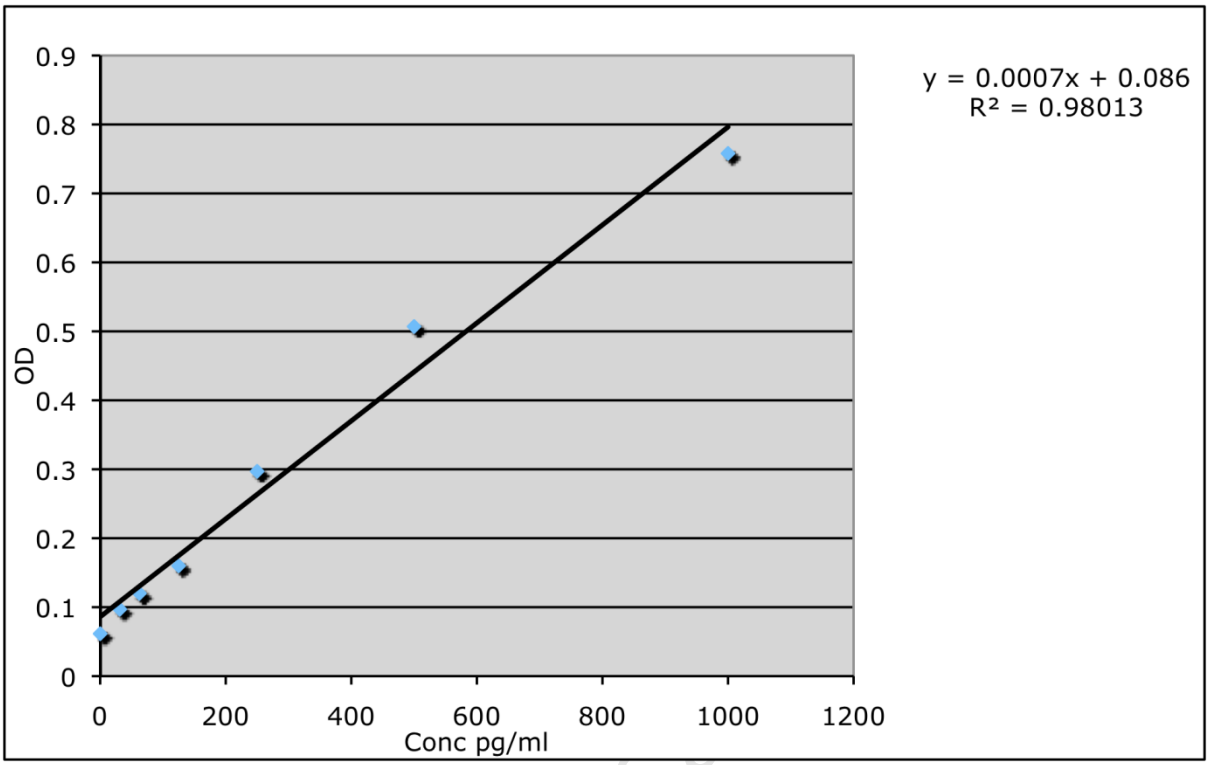
Appendix 5: Standard curve of IL-21



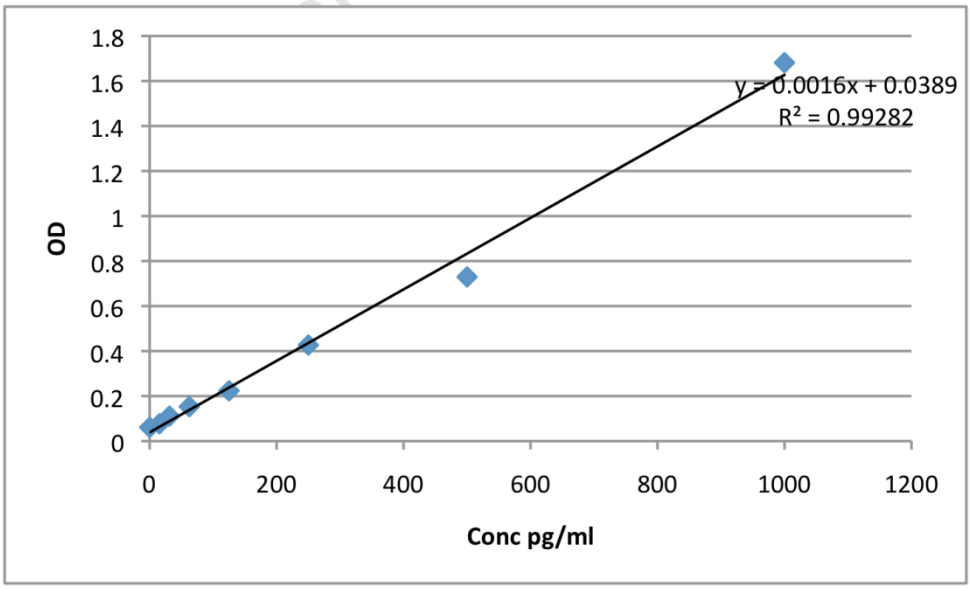
Appendix 6: Standard curve of IL-23 ready set go ELISA



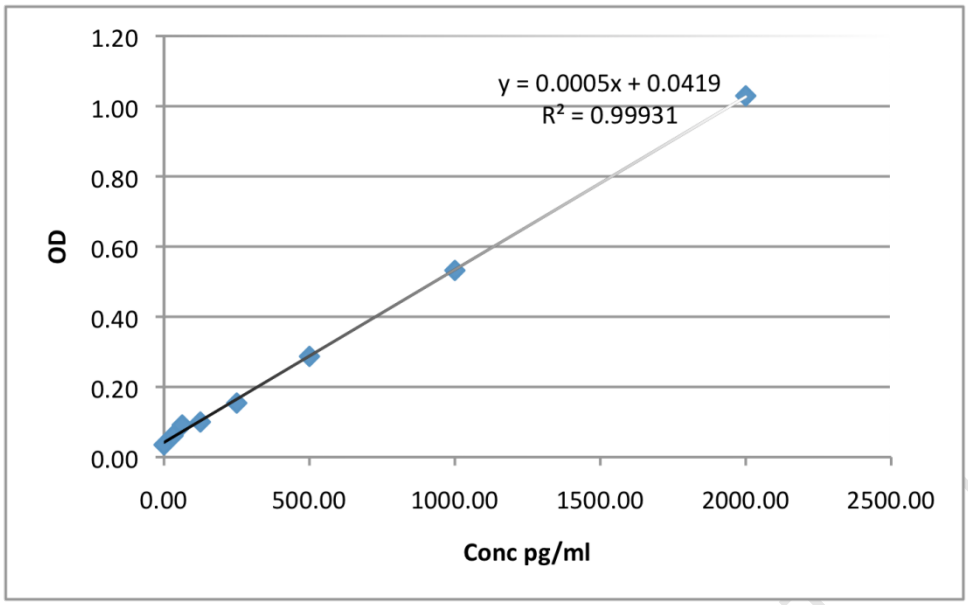
Appendix 7: Standard curve for TGF-B1 duoset ELISA



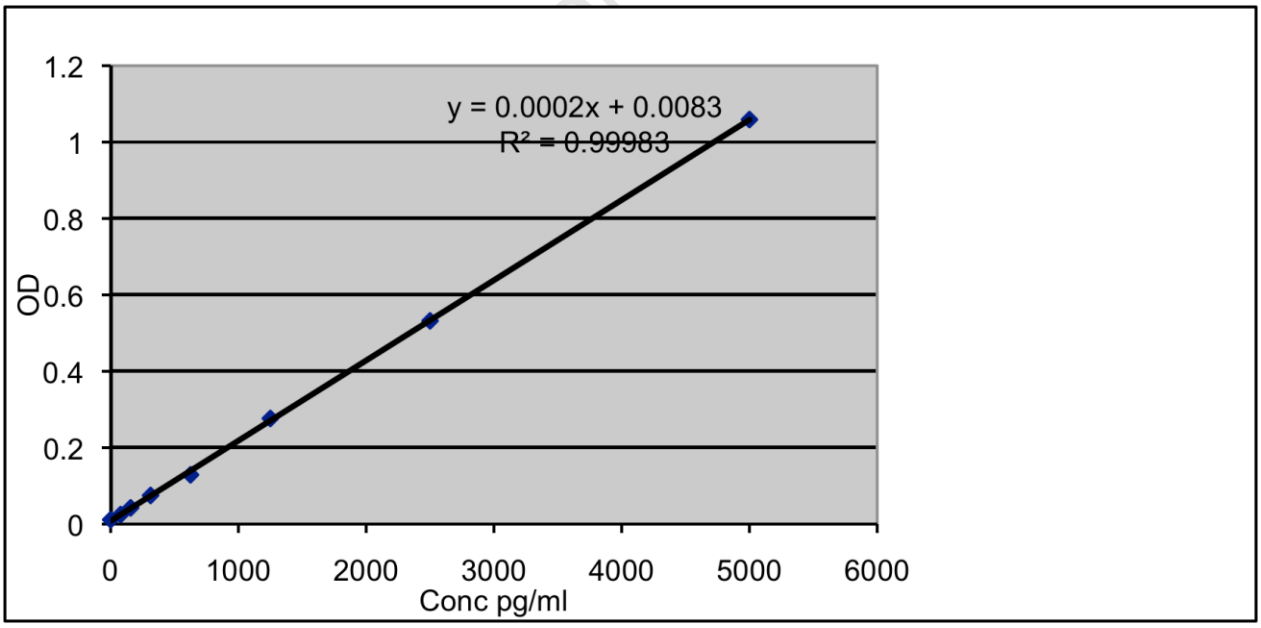
Appendix 8: IL-22 Quantikine ELISA standard curve



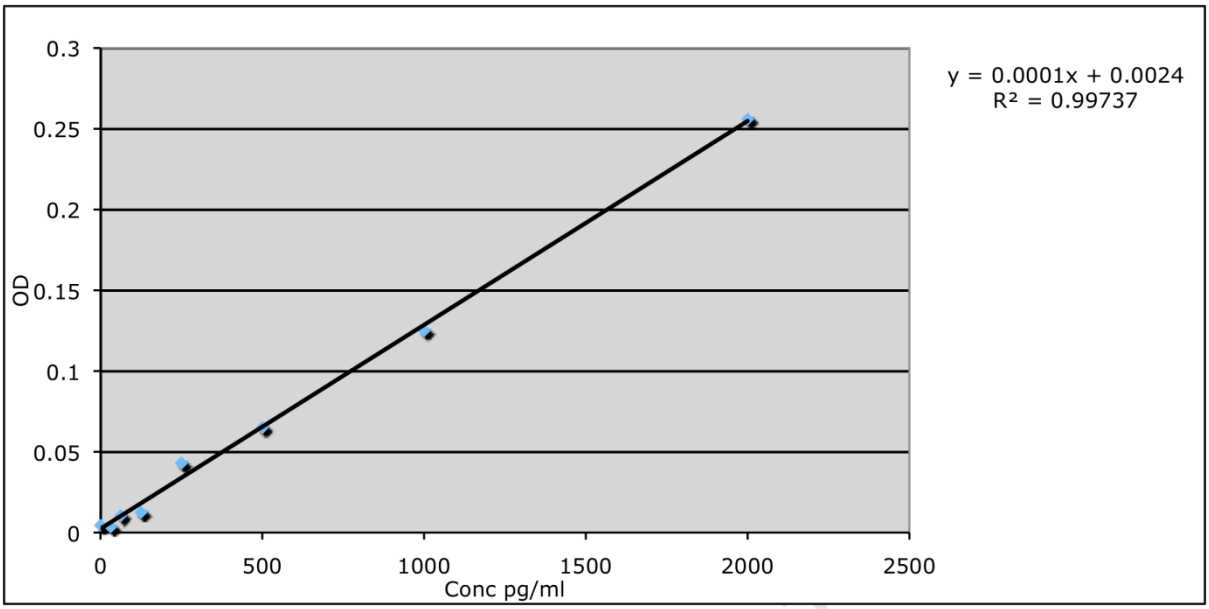
Appendix 9: IL-19 Quantikine ELISA standard curve



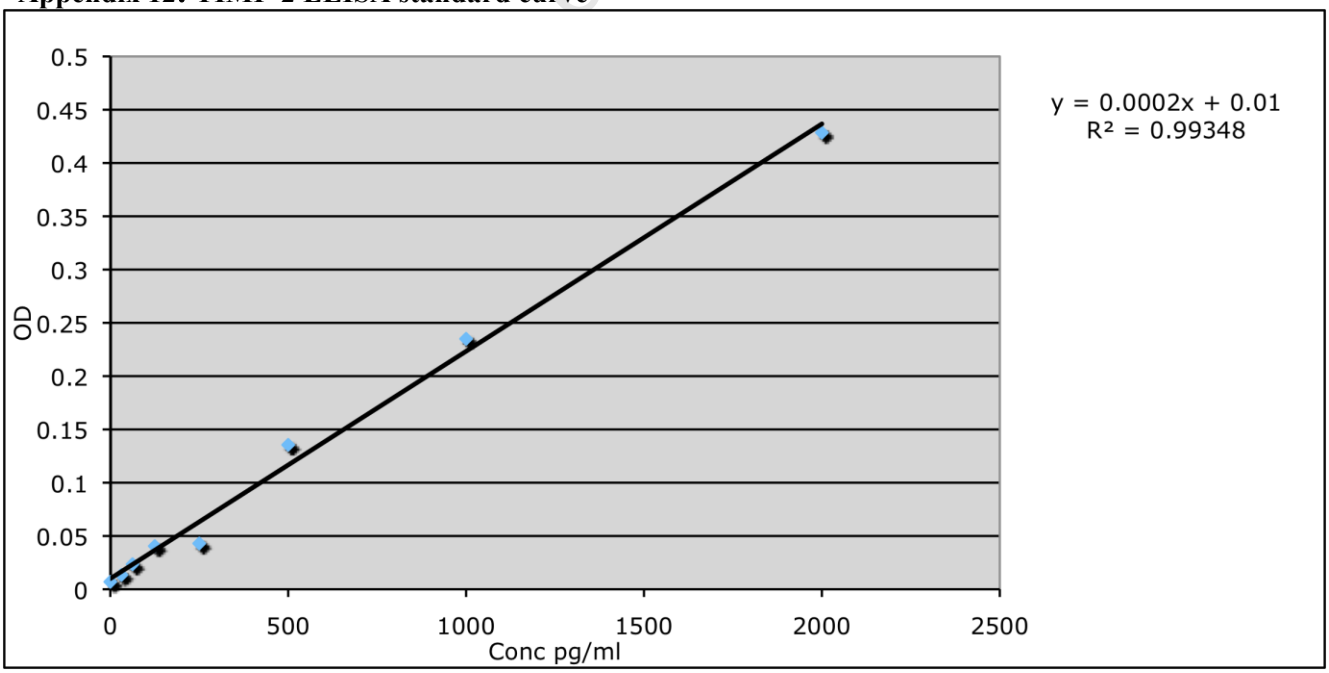
Appendix 10: MMP-10 Quantikine ELISA standard curve



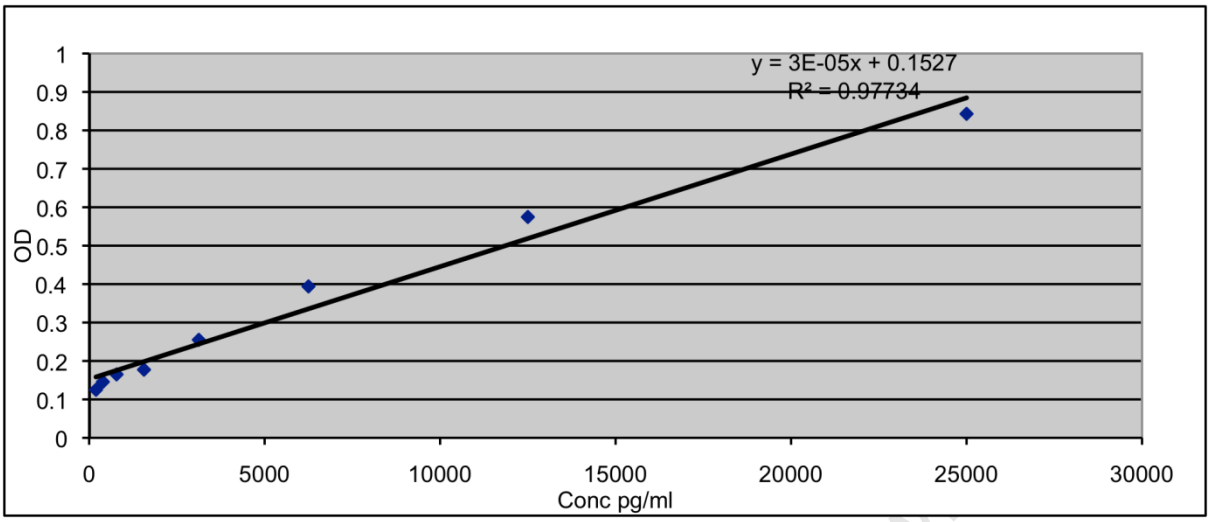
Appendix 11: TIMP-1 ELISA standard curve



Appendix 12: TIMP-2 ELISA standard curve



Appendix 13: MMP-9/TIMP-2 ELISA



Appendix 14: MMP-2/TIMP-1 ELISA